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Characterization and Analysis of Neural Progenitor Cells from Primary Progressive Multiple Sclerosis Patients

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Abstract

Primary progressive multiple sclerosis (PPMS) is a chronic demyelinating disease of the central nervous system (CNS) currently lacking any effective treatment. Promoting endogenous brain repair offers a potential strategy to halt and possibly restore neurologic function in PPMS. To understand how the microenvironment within white matter lesions plays a role in repair I focused on neural progenitor cells (NPCs) since they have been found within lesions and influence oligodendrocyte progenitor cell (OPC) maturation. To better understand the cellular nature of NPCs in PPMS I developed induced pluripotent stem cells (iPSCs) from blood and skin samples of PPMS patients and age matched non-disease controls. Using these lines I determined that NPCs from PPMS cases provided no neuroprotection against active CNS demyelination and failed to promote OPC maturation whereas NPCs from age-matched control cell lines did so efficiently. I determined that NPCs from PPMS patients displayed hallmarks of cellular senescence, inhibiting their proper functioning. Cellular senescence is a form of adaptive cellular physiology associated with aging. Cellular senescence causes a pro-inflammatory cellular phenotype that impairs tissue regeneration, has been linked to stress, and is implicated in several human neurodegenerative diseases. Senescent NPCs were identified within white matter lesions of human progressive MS autopsy brain tissues. Expression of cellular senescence genes in PPMS NPCs was found to be reversible by treatment with rapamycin which then enhanced PPMS NPC support for oligodendrocyte differentiation. A proteomic analysis of the PPMS NPC

secretome identified high mobility group box-1 (HMGB1), which was found to be a senescence-associated inhibitor of oligodendrocyte differentiation. Transcriptome analysis of OPCs revealed that senescent NPCs induced expression of epigenetic regulators mediated by extracellular HMGB1. Lastly, I determined that NPCs are a source of elevated HMGB1 in human white matter lesions. Based on these data, cellular senescence contributes to altered NPC functions in demyelinated lesions in MS. Moreover, these data implicate cellular aging and senescence as a process that contributes to remyelination failure in progressive MS which may impact how this disease is modeled and inform development of future myelin regeneration strategies.

Characterization and Analysis of Neural Progenitor Cells from Primary Progressive Multiple Sclerosis Patients

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Characterization and Analysis of Neural Progenitor Cells from Primary Progressive Multiple Sclerosis Patients

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Table of Contents

List of Figures and Tables

Chapter 6: Discussion and Future Directions

Chapter 1: Multiple Sclerosis (MS) and Remyelination

I. Diagnosis, Clinical Forms, and Treatments for MS

Multiple sclerosis (MS) was first well defined by Jean-Martin Charcot (1825-1893), a French neurologist at the Hôpital de la Salpêtrière in 1868, where he named the disease “la sclérose en plaques”, literally meaning a “plaque-like sclerosis” [1]. We now know that the presence of sclerotic plaques in the brain pathology from patients who had this disease are inflamed and demyelinated areas. Brain lesions remain one of the bases for diagnosis for MS, but identification of these lesions has been modernized with imaging techniques such as magnetic resonance imaging (MRI).

MS is currently the most prevalent chronic inflammatory disease of the central nervous system (CNS) among young adults, affecting approximately 2.5 million people worldwide [2]. The disease is characterized by chronic lesions or plaques of demyelination found throughout the brain and spinal cord. The demyelination is a result of death of the myelinating cells, the oligodendrocytes (OLs), which renders axons susceptible to injury, without trophic support, and without their electrically insulating layer. Axons that are typically myelinated, and then become demyelinated with the disease, have deficits in saltatory conduction. At the earlier stages of disease, patients most often present with disrupted vision, tingling in their limbs which can be accompanied with limb weakness, or ataxia. It is important to point out that the symptoms a patient first experiences will vary based on the location of lesions, and the complete disease course for any patient is highly individualized and will vary depending on the subtype of MS (Fig. 1.1) [3].

There is currently no single test that can accurately diagnose MS. It is generally the clinical repercussions of the disease that coax someone to come to the clinic. In corroboration with patient history and neurological exam, after the presence of symptoms, physicians rely on the use of MRI to identify lesions that are disseminated in time and disseminated in space, which have been the most important criteria in diagnosing MS [4]. In July of 2000 the International Panel on the Diagnosis of MS met and produced what is now called the McDonald Criteria for diagnosing MS

[5]. This set of criteria has been evolving and is updated every couple of years due to new discoveries and methodologies for diagnosis, and has been most recently updated in 2017. Brain and spinal cord MRI still remain the most useful test that aid in the diagnosis of MS. In order to distinguish MS from other acute demyelinating conditions, such as acute demyelinating encephalomyelitis, repeated imaging is necessary to determine if CNS lesions are disseminated in space and time [3]. CNS lesions in MS are typically multifocal white matter lesions in characteristic locations, including: periventricular, juxtacortical (next to the cortex), and in the spinal cord, close to the pial surface [6]. Examination of the cerebrospinal fluid (CSF) for oligoclonal banding, which is the presence of immunoglobulins, is also used to diagnose MS [7]. The observation of immunoglobulins in the CSF is indicative of the immune system generating antibodies against self, but it is not specific to just MS, therefore CSF testing needs to be used in conjunction with other methods of diagnoses [8, 9]. Breakdown of the myelin peptide, myelin basic protein (MBP), has also been found to be present in the CSF of patients with MS, but its presence does not increase accurate diagnosis [10]. MS is the most common condition that induces demyelination, but there are other conditions that can damage myelin including viral infections, toxin poisoning, and hereditary disorders, therefore most of these need to be eliminated before a diagnosis of MS can be achieved [11].

There are various subtypes of MS including: relapsing-remitting MS (RRMS), secondary-progressive MS (SPMS), progressive relapsing, and primary progressive MS (PPMS) (Fig. 1.1). RRMS represents 80-85% of all MS cases, is typically diagnosed in young adults (20-30 years of age), and is more common in women with a ratio of 3:1 [12]. The disease is characterized by episodic attacks of new or escalating neurological symptoms called “relapses”, which are typically followed by periods of remittance, where the disease spontaneously resolves. Symptoms generally start with sensory disturbances, such as double vision, which then worsen over time to include limb weakness resulting in clumsiness. Over time patients will be encumbered with fatigue, display cognitive impairment, and the disease often culminates in progressive disability

leading to quadriparesis [13]. The National MS Society currently predicts that 25 years after RRMS diagnosis 90% of patients will develop SPMS, generally around 40 years of age [14, 15]. SPMS is diagnosed when the patient experiences a worsening disease condition without the periods of remission. Unfortunately, when disease advances to the progressive phase current immune-modulatory therapies fail to provide any clinical benefit and do not slow down the worsening development of the disease (Fig. 1.2).

PPMS is the most aggressive form of MS and represents approximately 10-15% of all MS cases. These patients are typically diagnosed in their 40s and their disease is described as a gradual but progressively declining clinical course, which often presents as an upper-motor-neuron syndrome of the legs [14, 16]. This disease then gradually worsens into quadriparesis, with cognitive decline, and eventually cerebellar, bowel, bladder, and sexual dysfunction [13]. Unlike relapsing forms of MS, the immune system seems less involved in the brains of PPMS patients [16], which makes the current immunomodulatory therapies useless in treating these patients (Fig. 1.2). Salient differences between PPMS and RRMS will be discussed further in this chapter.

The majority of MS cases, specifically the RRMS subtype, are believed to be driven by the immune system categorizing them as an autoimmune disease. Helper and cytotoxic T cells have been found in MS lesions [17], and have been found to be reactive to myelin self-antigens - which are used as a diagnostic for MS. It is the development of autoimmunity directed against myelin that is currently thought to bring about the eventual destruction of myelin in MS. RRMS patients, in particular, have been found to have myelin-reactive T cells that exhibit an activated phenotype, measured by an increase in proliferation. In contrast, T cells from healthy controls exhibit a naïve phenotype, with a significantly lowered amount of proliferation [18]. Activated T cells can cross the blood-brain barrier (BBB) through a step-wise process called extravasation. The BBB in MS patients becomes abnormally permeable to immune cell infiltration before inflammatory demyelination in MS [19]. Known T cell targets include myelin antigens, such as myelin basic

protein (MBP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP) [13]. In addition, the most common DNA sequence variants in patients with MS are those in the human leukocyte antigen (HLA) locus, which code for the cell-surface proteins responsible for the regulation of the immune system [20]. To support the T cell-driven theory of MS, a similar experimental disease, called experimental autoimmune encephalomyelitis (EAE), can be induced in rodents and primates by immunization with myelin antigens. EAE leads to inflammatory CNS demyelination driven by myelin-reactive T cells. Current effective treatments for RRMS focus on the activation and migration of immune cells to abate the T cell response, such as interferon (IFN)-β (Betaseron), which decreases the expression of T helper cells, and natalizumab (Tysabri) which prevents T cell infiltration across the BBB by blocking adhesion to the neurovascular endothelium [21].

B cells, the professional antigen presenting cells of the immune system, have also been implicated in MS, as B cell-depleting antibodies have been found to limit MS lesion formation and clinical disease activity [22]. B cells also produce antibodies, which have been found in lesion areas, but also produce cytokines, in turn becoming pathogenic [23]. It is becoming increasing clear that B cells play an important role other than antibody production in MS, as the amount of antibodies are largely unchanged after B cell depletion [24]. Their actions in the development and progression of MS are still largely unknown. Ocrelizumab (Ocrevus) is currently the only FDA approved therapy for the treatment of progressive forms of MS, which works by targeting B cells [25]. Currently the extensive body of work on the immune system in MS, including the diversity of cells and cellular response in MS is complex, and while important, exceeds the scope of this thesis which is focused on progressive MS.

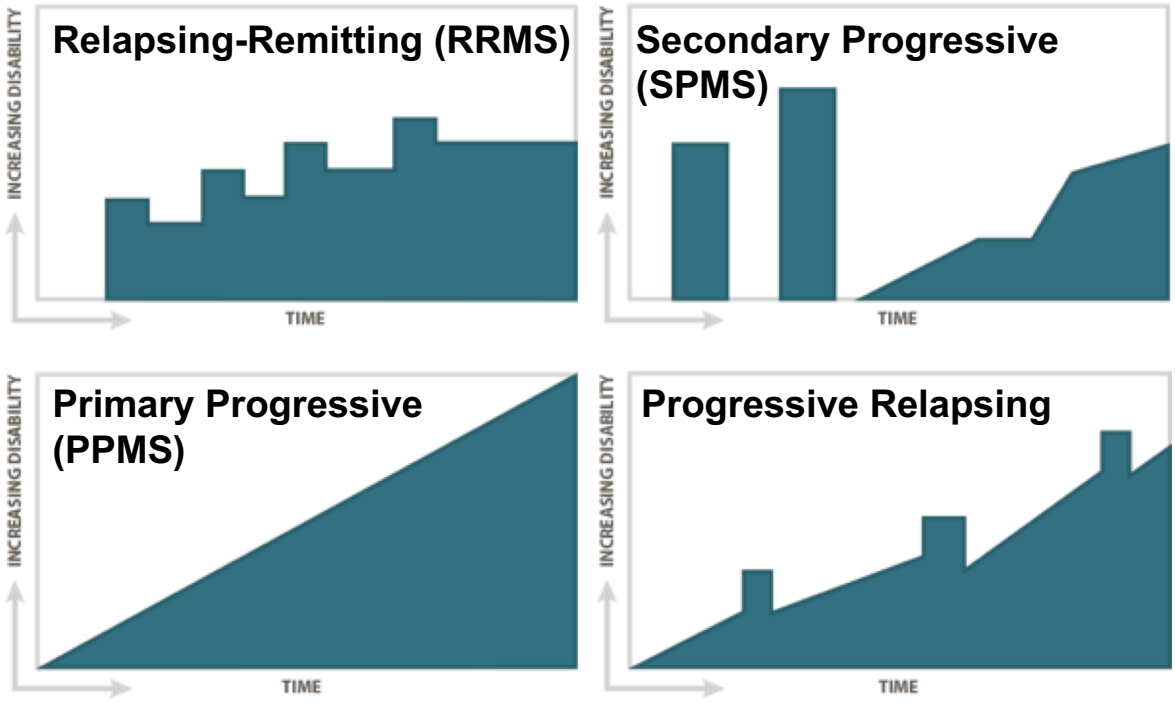

Figure 1.1. Clinical disease courses in the various subtypes of MS. Representative graphs of the clinical presentations of the subtypes of MS. Y axis depicts the increase in disability and the X axis represents time. Panels are labeled based on the subtype of MS they are representing. Figure from the National Multiple Sclerosis Society (https://www.nationalmssociety.org/What-is-MS/Types-of-MS).

Currently all treatments for MS predominately focus on the immune system. Corticosteroids, such as intravenous methylprednisolone, are initially administered to dampen the inflammation that occurs during a MS relapse [26]. It has been proposed that corticosteroids reduce the expression of adhesion molecules [27], which helps prevent the extravasation of damaging immune cells into the CNS, while also reducing T cell activation and increasing expression of transforming growth factor (TGF)-β which is detectable in the CSF [28]. Corticosteroids provide a shorter recovery time from relapses, but show no overall long-term benefit [29].

The first disease-modifying treatment (DMT) for MS, approved by the Food and Drug Administration (FDA) in 1993, is interferon (IFN)-β (Betaseron) (Fig. 1.2). Currently, there are five formulations of the drug that have been developed to treat RRMS. IFN-β has been shown to increase the production of anti-inflammatory cytokines, such as IL-10 and IL-4 [30], decrease the production of pro-inflammatory cytokines, such as IL-17 [31], and limit immune cell trafficking across the BBB [32, 33]. In placebo experiments IFN-β was shown to be effective in reducing the relapse rate in patients with RRMS, but does not alter the progressive disease activity [34]. IFN-β has no effect on the time it takes for RRMS patients to develop SPMS, instead it only decreases rate and duration of relapses, and provides no measurable benefit for PPMS patients [35].

Glatiramer acetate (Copaxone) is another immunomodulatory drug made up of a mixture of peptides that are found in myelin basic protein (MBP) (Fig. 1.2). The mechanisms of how this drug works are not fully understood, but it is believed to be able to promote the shift of pro-inflammatory T cells to regulatory T cells that suppress an inflammatory response [36]. In clinical trials, it prolongs the time to additional relapses and reduces the risk of new lesions, but does not alter the development of SPMS [37].

Natalizumab (Tysabri) was the first monoclonal antibody that was approved for RRMS, which acts by targeting a receptor (α4 integrins) found on activated immune cells, preventing them to migrate into the CNS (Fig. 1.2) [21]. Clinical trials have shown that it reduces the number of

clinical relapses and treatment resulted in fewer new and enlarging lesions [38]. Although natalizumab has been characterized as effective in the reduction of relapses, as well as new lesion formation, it leaves the CNS with no immune protection, which may lead to the development of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease caused by the John Cunningham (JC) virus [39]. In a study surveying a large, multinational MS population, the JC virus antibody was found to be in 57.1% of the population, eliminating over half of the total MS population that could safely take this drug [40].

Fingolimod is a sphingosine 1-phosphate receptor (S1PR1) modulator, binding to this receptor expressed on immune cells, leading to its internalization and downregulation. The internalization of S1PR1 on T cells prevents their release from the lymph nodes and thus dampens the immune responses [41]. The mechanism of action of fingolimod may also be CNS mediated as S1PR1 expression on astrocytes has been shown to be necessary for the clinical benefit of treatment in an animal model of disease [42, 43]. Clinical trials have shown that with early treatment it can reduce the risk of disability progression and decreases the number of new or enlarging lesions [44]. Interestingly, fingolimod has been shown to have potentially neuroprotective effects in the EAE model of MS [45] and may promote the differentiation of oligodendrocyte progenitor cells (OPCs) into mature, myelinating oligodendrocytes [46, 47].

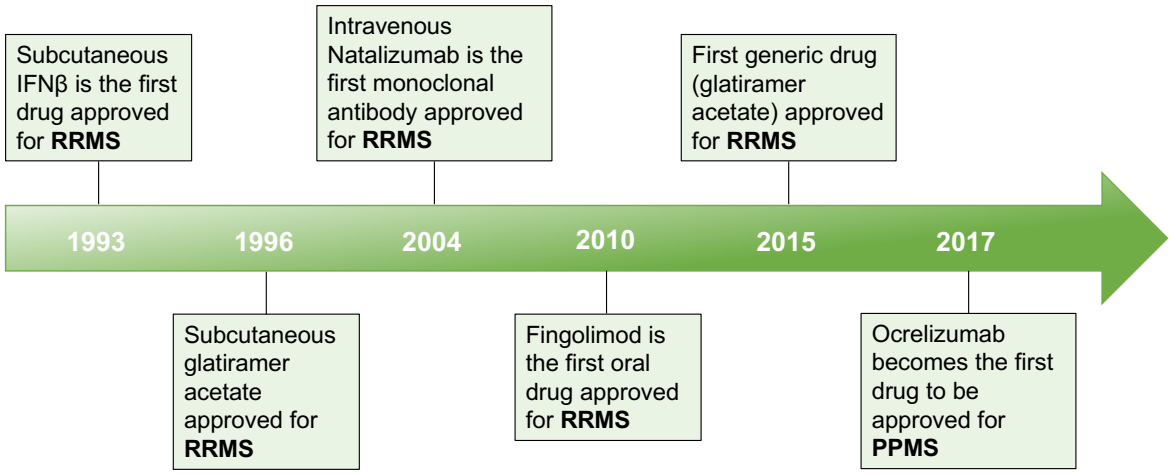

Figure 1.2. Timeline of drug developments in the treatment of multiple sclerosis. Milestones made in drug approval for the treatment of MS by the FDA. RRMS, relapsing-remitting multiple sclerosis; PPMS, primary progressive multiple sclerosis. Figure adapted from Tintore et al., 2018 [48].

Over the past couple years clinical studies have determined that the earlier DMT treatment can begin in patients with RRMS (within 5 years of the diagnosis) the more effective these therapies are at slowing the progression of disease into the progressive phase (i.e. SPMS), yet treatment does not reduce the progression of disability once SPMS manifests [49]. In contrast to RRMS, where there are many anti-inflammatory and immunomodulatory therapies that help with the number and severity of relapses, there are very little treatment options available for PPMS or SPMS that are effective. In March of 2017 the first drug for the treatment of PPMS was approved by the FDA, ocrelizumab, where it only reduced the risk of progression by 24% in patients compared with placebo controls [25] (Fig. 1.2). I will discuss these findings further in the next section on primary progressive MS. Due to the lack of effective treatments for SPMS and PPMS targeting repair strategies via remyelination is an ideal way to develop future treatments for MS.

II. What causes MS?

MS is not considered an inherited genetic disease, but the use of genome-wide association studies (GWAS) has identified over 100 genetic regions that are associated with the risk of developing MS [50]. These extensive genetic surveys have revealed the presence of "genetic susceptibility" for MS but no single genetic locus as a culprit. The majority of the MS-associated candidate genes are related to immunology and T cell function, which are often common to other autoimmune diseases, such as rheumatoid arthritis [51]. These include human leukocyte antigen (HLA) genes, which are those that code for cell surface antigen-presenting proteins that modulate immune system responses. Polymorphisms in these genes are believed to account for 20-60% of the genetic susceptibility in MS [52]. Another gene candidate identified is apolipoprotein E (ApoE), which is synthesized in the CNS, has immunomodulatory effects, and is involved in lipid and cholesterol transport as well as brain development. A meta-analysis of 6,977 subjects proposed a mutation in ApoE increased MS risk [53]. However, MS is not carried as a Mendelian trait, but there is a higher disease concordance rate within families and different ancestral groups [54, 55]. A Canadian twin study, published in 2003, showed a concordance rate of MS to be 25.4% between monozygotic twin pairs and 5.4% in dizygotic twins, which suggests a genetic component to the disease, but did not account for environmental factors outside of utero [56]. There has been no one study which shows a complete dependence on genetics in the development of MS, instead it is believed to be caused by a multitude of factors. MS is a multifactorial and complex condition, with a heterogeneity of subtypes, and based on current GWAS it is not only caused by one or two genetic mutations, but genetics may increase susceptibility.

Environmental influences such as latitude, vitamin D deficiency, viral or bacterial infections, and obesity have been thought to be implicated in the cause of MS. Those living at higher latitudes have a higher prevalence in developing MS, which is largely believed to be due to UV exposure and vitamin D exposure [57]. Another study demonstrated, by compiling data

from other studies, that insufficient UV light of the correct wavelength, which enables vitamin D synthesis, increases the potential of MS prenatally. This study also demonstrated the 'month of birth' effect, where those born during winter months have a reduced risk of MS, while those born in the spring have an increased risk [58]. Maternal vitamin D levels during pregnancy affect the immune system of the fetus, which may alter the risk of developing MS [59].

For many years studies have tried to determine if a single virus could represent a causative agent for developing MS. These investigations have yielded no pathogen link to MS, although potentially compelling associations with several agents have been suggested. One aspect of this disease where viruses have been implicated is in the severity of relapses [60]. Viral infections of the CNS can cause inflammation and damage to oligodendrocytes. The damaged fragments of myelin can be recognized by autoreactive T cells that can in turn trigger more inflammation and myelin destruction [61]. An additional potential mechanism is that infections outside of the CNS can activate T cells with a foreign peptide similar to that of a myelin peptide, which is termed "molecular mimicry" [62]. For example, molecular mimicry between myelin basic protein and the Epstein-Barr virus (EBV) latent antigen, EBNA1, has been well documented [63]. Interestingly, human data has demonstrated that MS risk is extremely low in individuals not infected with EBV, and EBV infection increases the risk of MS [64, 65]. EBV-specific T cells have been found to be expanded in the CSF of patients with MS [66]. However, EBV, or other viruses, such as influenza, are not the sole cause of MS, but may play a role in the severity of disease course and relapses.

The cause of MS is not known. It is widely accepted that MS is a multifaceted, complex disease. There is no single factor implicated in the cause of MS, instead it may be caused by interaction through the various factors mentioned above, including genetic susceptibility and environmental instances. Based on the varying subtypes of MS and the constellation of symptoms per patient, causal pathways for disease are likely to differ from individual to individual.

III. Primary Progressive Multiple Sclerosis (PPMS)

The clinical features of PPMS are vastly different from the more common RRMS. Patients with PPMS, instead of encountering relapses, develop a disease course of chronic unremitting worsening disability. This form of MS affects 10-15% of the MS patient population and differs from RRMS in that it has no gender bias [67, 68]. What is most striking about PPMS is that patients are usually diagnosed in their 40s or 50s which is significantly later in later than RRMS which is typically diagnosed in the late 20s [14]. Interestingly, the majority of RRMS patients, after 20-25 years of diagnosis, advance into a secondary-progressive disease course (SPMS), which is also characterized by a clinical phenotype similar to primary progressive disease; persistent development of symptoms without relapses [69]. Both PPMS and SPMS are typically diagnosed around the same age, suggesting that processes in aging may contribute to the development of progression in MS [70]. For instance, eight years after presentation and diagnosis of progressive MS, patients generally require assistance for walking, and typically after 18 years patients are wheelchair-bound [67]. Most commonly, patients with PPMS present with visual loss but also spinal syndrome, reflected in symptoms that include spastic paraparesis and cerebellar dysfunction [71].

What remains a consistent feature of progressive forms of MS is the presence of white matter lesions, along with atrophy and cortical demyelination, while the essential element is “a gradual nearly continuously worsening baseline with minor fluctuations but no distinct relapses” [72]. Currently, PPMS is diagnosed based on positive MRI lesions, especially in the spinal cord, a delayed visual evoked response, and identification of oligoclonal bands in the CSF [73]. There is no one blood or CSF marker that can accurately diagnose PPMS, instead, like RRMS, it is based on the symptomology experienced by the patient along with MRI lesion evidence. Progressive MS is accurately diagnosed post-mortem by analysis of white matter lesions in corroboration with patient symptoms. Actively demyelinating white matter lesions, as defined by the increased presence of macrophages at the border in and around the lesion area, are more

frequently found in RRMS. Lucchinetti et al. identified and described four different patterns of lesions found in MS [23]. Patterns I and II share similar features of active demyelination associated with the presence of T cells and macrophages. The difference is the presence of immunoglobulin deposits at the lesion site, found exclusively in pattern II. Both pattern I and II lesions are associated with small veins and venules with active myelin destruction in the plaque center. Pattern III lesions are composed mainly of T cells, macrophages, and activated microglia without the presence of immunoglobulin deposits. The borders of these types of lesions are ill defined, and demonstrate spreading into the surrounding white matter. Lesion patterns I-III are associated with shadow plaques, indicating remyelination, and are distributed homogenously in RRMS. Lastly, pattern IV lesions are defined by primary oligodendrocyte degeneration, similar to a viral infection or toxin and not associated with autoimmunity [23]. It is unknown whether these lesion patterns change over the disease course, but pattern IV lesions are exclusive to PPMS [16].

Inactive or chronic active MS lesions are characterized by a rim of microglia and sometimes macrophages around the lesion area, and are more common in PPMS [74]. Interestingly, inflammation from invading macrophages, such as T cells, is less pronounced in progressive MS, suggesting that the inflammation is trapped within the blood-brain barrier [75]. Slowly expanding lesions account for approximately half of all lesions found in progressive MS [75, 76]. The lesion center is an area of complete demyelination with evidence of profound axonal loss and significant astrocytic scarring. Lesions have also been noted to be surrounded by activated microglia, which are also scattered diffusely surrounding normal-appearing white matter [77]. Besides white matter lesions, another prominent neurological sign in MS patients is the loss of overall brain volume [78]. During the progressive stage of the disease there is a gradual expansion of pre-existing white matter lesions and widespread cortical demyelination, although these changes occur with only mild apparent blood-brain barrier damage [79], making this form

of MS significantly different from RRMS, which is primarily T cell mediated with prominent BBB breakdown [17, 80].

Histological analysis of demyelinated lesions has found oligodendrocyte progenitor cells (OPCs) present within chronic lesions [23, 81]. Since these cells have the capability of differentiating into mature, myelinating oligodendrocytes, they offer the potential for brain repair, though these cells within lesions exhibit very little to no remyelination activity. Remyelination in MS is indicated through the presence of “shadow plaques”, which are partially remyelinated lesions and can be identified with luxol fast blue staining [23]. Analysis of post-mortem brain tissue found that remyelination in MS patients is variable from patient to patient, yet is significantly sparse in progressive MS cases [16, 82, 83]. The mechanisms of myelin repair in patients with progressive MS remain elusive and represent a field of intense study. More recently age has been proposed as a natural limit on CNS myelination which may have implications for understanding the disease-related processes of limited remyelination in MS.

Current disease modifying therapeutics used to treat RRMS have little to no therapeutic effect in progressive MS. There are no significant treatments that reverses, stops, or even slows the progressive disability once it has become established, but for the first time in March of 2017 a drug for the treatment of PPMS was approved by the FDA (Fig. 1.2) [84]. Ocrelizumab is an immunosuppressive drug, a humanized monoclonal antibody, that binds to CD20, only found on B cells, and selectively kills them by causing antibody-dependent cell-mediated cytotoxicity. B cells have been found to contribute to MS through antigen presentation, the production of antibodies, and cytokine secretion. In progressive MS B cells have been found in the meninges of patients, where they could be furthering chronic demyelination and inflammation [85, 86]. A recently published trial, performed in only patients with PPMS, indicates that ocrelizumab was associated with lower rates of clinical and MRI progression compared to those on a placebo [25]. Overall, out of the PPMS patient population selected, 32.9% taking ocrelizumab versus 39.3% taking placebo had confirmed disability progression after 12 weeks. In addition, the percentage

of brain-volume loss was 0.9% with ocrelizumab and 1.09% with placebo [25]. This still leaves significant room for improvement in drug therapies to slow progression in these patient populations with progressive MS, and suggests that a multifaceted therapy may be best, such as a combination of immune suppression and remyelination.

Despite the increasing knowledge on progression in MS there are still many questions that remain unanswered, such as the underlying mechanisms that drive progression. Understanding the exact mechanisms involved in the progressive forms of MS will aid in the development of new therapeutics that could target multiple aspects of the disease process. One way to model how remyelination can be controlled and how progression develops is through the use of animal models.

IV. Animal Models of Central Nervous System (CNS) Demyelination

Since MS is a complex human disease, there is no single animal model that can capture exactly what goes on in human MS. Nevertheless, mouse models have become a tremendous resource toward understanding how specific aspects of the immune system function and how demyelination occurs in MS [87]. Animal models have been used to study disease development, test novel therapeutic approaches, and provide a convenient source of CNS tissue in which to study demyelination. Currently, the most widely adopted animal models of CNS demyelination are: experimental autoimmune encephalomyelitis (EAE), virus induced demyelination (i.e. Theiler's murine encephalomyelitis virus (TMEV) infection), and toxin-induced models (cuprizone).

The most commonly used animal model of MS is EAE. This T cell dependent model of CNS demyelination results from an induced autoimmunity evoked by immunization with components of myelin. In the 1930s, in an attempt to produce encephalomyelitis Rivers et al. immunized monkeys with rabbit brain extracts which were found to induce paralysis associated with immune cell infiltration and demyelination in the brain and spinal cord [88]. Now, EAE is

induced by injection of specific myelin peptides, specifically antigenic epitopes of myelin proteins, such as: myelin oligodendrocyte glycoprotein (MOG) (35-55) peptide, MBP (87-99) peptide, or proteolipid protein (PLP) (139-151) which are emulsified in complete Freund's adjuvant (CFA) [89]. While many protocols for induced EAE have been developed, injection of pertussis toxin, which has been suggested to transiently enhance the permeability of the BBB, has been found to increase the responsiveness of animals to the immunization of self-antigens [90, 91]. EAE in mice is characterized by an ascending paralysis, beginning with tail atonia, followed with hind limb paresis and paralysis which can advance to involve forelimb paralysis (quadriplegia). Based on the genetic background of the mice (SJL/J, C57BL/6, NOD), and on the type of immunization (protein vs. peptide) the EAE course will vary. Most commonly, EAE induced using MOG peptide (35-55) in C57BL/6 mice is well characterized as a monophasic disease course, without relapses. The disease is instigated by autoreactive CD4+ T cells that primarily cause demyelination in the spinal cord, unlike human MS, where lesions are primarily in the brain [92]. Other models of EAE include MBP-derived peptides or PLP administered to SJL/J mice [93, 94] which induce spontaneous relapses and have utility as a model of RRMS.

The use of EAE has benefited research and patients with MS, as three of six approved MS therapeutics showed results with this model: glatiramer acetate, mitoxantrone, and natalizumab [92]. However, EAE is not the perfect model as it does not recapitulate the same immune mechanisms seen in patients [95]. For example, the lesions in EAE are primarily in the spinal cord whereas lesions are more prevalent in the brains of patients, there is difficulty in reproducibility of EAE severity, and there is inherent difficulty in studying remyelination processes because lesions form sporadically and not in predictable locations. A pharmaceutical example would be the use of a TNF neutralizing antibody. In EAE mice suppression of TNF was found to ameliorate disease, while in human trials, unexpectedly, it was found to worsen the disease, and therefore further study has been discontinued [96, 97]. EAE also provides limited insight into understanding the disease-related processes of progression in MS or PPMS pathology as EAE

models are primarily T cell dependent, while PPMS is not. In addition, mice with EAE do not display additional progression after immunization, instead they typically exhibit robust recovery and efficient remyelination, making them unsuitable for studying progressive forms of MS [92]..

Viruses have a long-standing association with MS [98]. Studies have suggested that viral infection(s) early in life, such as EBV, in genetically susceptible individuals may underlie induction of an immune-mediated attack against the CNS [99]. Viral infections of the CNS have been found to induce demyelination in mice, such as Theiler's murine encephalomyelitis virus (TMEV) and strains of the coronavirus mouse hepatitis virus (MHV) [100]. Through the use of viral models we have further elucidated possible mechanisms by which virus infections can lead to delayed CNS autoimmunity through epitope spreading [101]. Viral infection models of demyelination all rely on the activation of immune cells. Unlike MS the pathogenesis of TMEV-induced demyelination requires a persistent viral infection, and TMEV can only induce inflammatory demyelination in genetically susceptible strains of mice [102]. Interestingly, in TMEV infection, axonal damage precedes demyelination, which is believed to trigger the recruitment of T cells into the CNS, causing demyelination through what is called an "inside-out" mechanism [103]. Even though virus-induced demyelination in mice is significantly different from MS, these models have proven useful in testing new therapeutics for targeting axonal degeneration and immunosuppression.

Immune-mediated models of CNS demyelination all have limited utility for understanding how remyelination occurs and how it can be promoted. Targeting remyelination and neuroprotection holds the most promise in treating progressive MS. Development of additional models of demyelination that involve administration of toxins have enabled researchers to accurately time and localize sites of demyelination that allow for study of the endogenous process of remyelination. Currently, two models are extensively studied for these purposes: dietary consumption of cuprizone (Fig. 1.3), a copper chelator, and direct injection of lysolecithin into white matter tracts in the CNS. Cuprizone is fed to mice and causes dysfunction of mitochondrial complex IV which selectively kills oligodendrocytes because they are extremely susceptible to

oxidative stress [104]. Along with oligodendrocyte cell death, there is significant activation of astrocytes and microglia [105]. Cuprizone lesions are similar to human pattern IV MS lesions [23], which show very little to no T cell infiltration, and are characterized by significant oligodendrocyte death and innate immune activation (microglia) [81, 106]. White matter lesions in the cuprizone model are predictable and occur in highly myelinated areas such as the corpus callosum and cerebellum (Fig. 1.3). Based on the amount of cuprizone given in the diet, and the length of treatment, the amount of total demyelination will vary along with any accompanying behavioral deficits, which do not present as an overt phenotype and do not appear until at least 6 weeks of toxin treatment [107]. Although demyelination in the cuprizone model is extensive and widespread, once cuprizone is removed from the diet, new oligodendrocytes from the pool of OPCs begin to fully remyelinate the lesions [104]. Manipulation of this remyelination process has been adopted as a means by which to identify mechanisms on how remyelination occurs and how it can be promoted endogenously in progressive MS patients [108-110]. Although the cuprizone model is not a model to study autoimmune-mediated demyelination, it is widely used as an effective system to study the basic mechanisms that occur during demyelination and remyelination.

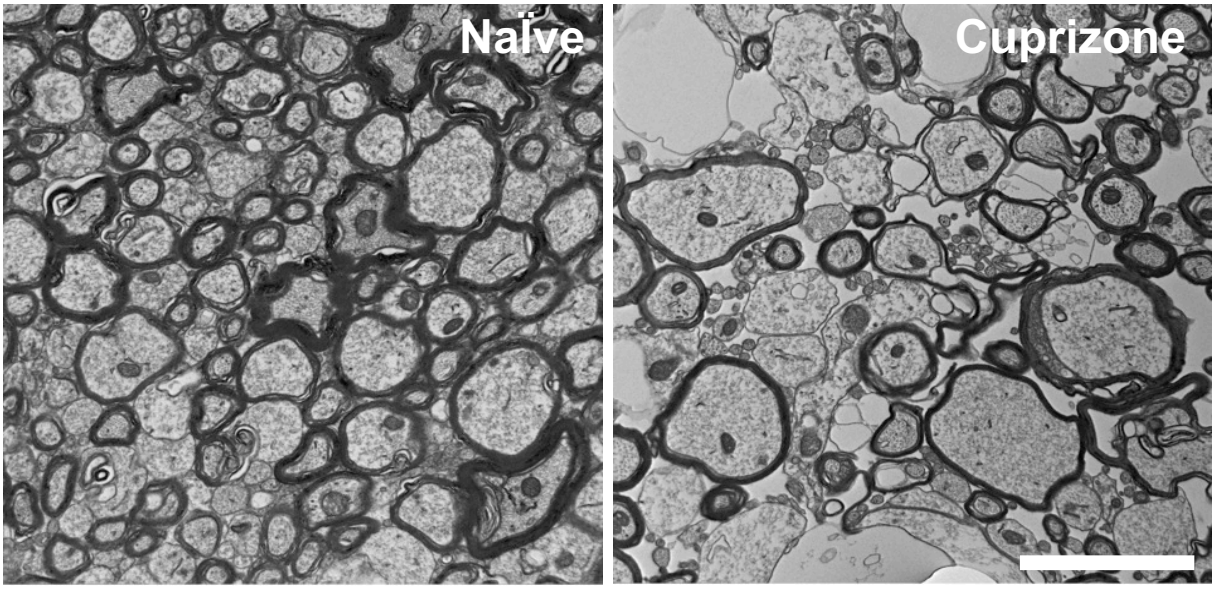

Figure 1.3. Representative electron micrographs (EM) of compact myelin in the corpus callosum of naïve wildtype mice (left) and mice treated with cuprizone (right). Six week old mice were fed cuprizone (0.2% w/v) for 4 consecutive weeks and myelin was imaged in the corpus callosum. Scale bar, 2 μm. Images from Nicaise et al., 2017 [111].

Injection of lysolecithin, an activator of phospholipase A2, into white matter tracts represents another model of CNS demyelination. When lysolecithin is injected it focally induces demyelination due to the primary toxic effects on oligodendrocytes [112, 113]. Focal injection produces a non-immune mediated, rapid and highly reproducible form of demyelination. This model allows for analyzing demyelination and remyelination as discrete events with "spatiotemporal predictability" [114]. Again, use of this toxin based model since does not involve engagement of T cells. When taken together, toxin based models provide systems to study the processes of de- and remyelination. Based on the research question, toxin models may provide the most appropriate mouse model for study, although it is important to note that no one animal model accurately replicates all of the features of myelin damage and immune system biology in human MS.

V. Endogenous Remyelination

Remyelination is mediated by a resident population of endogenous stem cells found within the brain throughout lifespan called oligodendrocyte progenitor cells (OPCs) and neural progenitor cells (NPCs) (Fig. 1.4). Both of these cell populations are widely distributed throughout the white and grey matter, and persist throughout adulthood [115]. These are both undifferentiated cell types capable of self-renewal, and both can give rise to mature oligodendrocytes if provided with the appropriate cues and signals. Neural progenitor cells arise from the adult subventricular zone (SVZ), located next to the lateral ventricles, and the subgranular layer of the dentate gyrus in the hippocampus [116, 117]. Even though the SVZ expands in response to demyelination and NPCs are found to migrate and reside within areas of demyelination, it is clear that remyelination fails [118]. The role of NPCs in remyelination failure in MS and in persistent demyelinated lesions in progressive MS are unclear [119]. The limited remyelination observed to occur in MS has limited long-term benefit due to the recurring inflammation in this disease, and compact myelin resulting from remyelination is much thinner than normal appearing white matter myelin [120]. For these

reasons the development of therapeutics which can promote healthy and permanent remyelination is a high priority. The ways in which NPCs contribute to impairing or promoting remyelination will be discussed in the next sub-chapter.

Oligodendrocyte differentiation occurs in a sequence of temporally defined steps that can be identified by expression of stage-specific markers and dramatic changes in cellular morphology. In development, oligodendrocyte differentiation is a highly regulated process that is controlled spatially and temporally through tightly controlled signals such as growth factors and extracellular matrix molecules. OPCs migrate more extensively compared to astrocytes and neurons. They arise from the ventricular zone in the embryonic brain and spinal cord and their domains are then defined through the formation of patterns [121]. In early development OPCs arrive ventrally, then followed by waves of dorsally derived cells [122, 123]. This long-range cellular migration of OPCs, from germinal zones, is critical to development and has been found to be regulated by transforming growth factor β (TGFβ), vasculature, and Wnt signaling, among other factors [122, 124]. Once they have migrated and populated the white matter, OPCs proliferate and mature. The eventual development of OPCs occurs in a series of stages dependent on extrinsic factors, such as the environment, and intrinsic factors such as epigenetics [124-126].

Immature OPCs have a bipolar morphology and express markers like platelet-derived growth factor α-receptor (PDGFR-α), NG2 proteoglycan, and A2B5 that distinguish these progenitors from more mature OPC lineage cells [127]. Once OPCs become mature enough to start generating myelin they can be identified by expression of myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG) [127]. Morphologically, maturing OLs become much more complex and extend numerous processes outward from the cell soma. *In vivo,* the number of myelinating processes vary depending upon the anatomical locus, but on average one oligodendrocyte can myelinate up to 40 different axons [128, 129].

Many factors have been shown to be important for the initial maintenance of OPCs in an undifferentiated state, while others have been identified as necessary for the differentiation of OPCs into mature oligodendrocytes. Bone morphogenic protein (BMP), PDGF, and fibroblast growth factor (FGF-2) are necessary in the developing CNS as they keep OPCs in a proliferative state and thus immature, ensuring a pool of cells for whenever de novo myelination is needed [128, 130, 131]. On the other hand, the thyroid hormone, triiodothyronine (T3), promotes differentiation of OPCs into oligodendrocytes [132]. Not only do growth factors have an effect on the proliferation and differentiation of OPCs, but these proteins do so by affecting intracellular signaling pathways. For instance, NOGO signaling via LINGO-1 has been found to inhibit oligodendrocyte differentiation, and Notch-1, which signals developmental fate of NPCs, also helps facilitate OPC migration and proliferation [133, 134]. In addition to these factors being involved in development, there are factors generated by the tissue damage in MS that also influence OPC fate, such as cytokines and chemokines, which impact the ability of OPCs to respond to signals and differentiate appropriately. For example, the cytokine TNF-α has been found to correlate with the severity of demyelinated lesions, and in EAE mice anti-TNF-α neutralizing antibody was found to provide neuroprotection [135-137]. However, remyelination in a TNF-α knockout mouse is significantly delayed, indicating that it also plays a role in CNS repair [109].

Both myelination and remyelination occur through the activation of genes that are regulated and mediated by transcription factors [138]. These epigenetic events require coordinated activation and/or inhibition to appropriately time OPC maturation for myelination and remyelination to occur correctly. For example, there is a multitude of evidence that demonstrates that histone deacetylase (HDAC) activity is necessary for inhibition of myelin gene expression to be removed [139]. Interestingly, a recent study applied transcriptomic analysis to examine differences in DNA methylation in the normal appearing white matter of MS patients [140]. This report identified gene networks and determined that genes linked to regulating oligodendrocyte

survival were downregulated, suggesting that perturbed signaling within OPCs may underlie failed OPC differentiation and impaired remyelination in MS [140]. The specific role of the myriad of factors that are known to alter remyelination in MS is unknown, and simply inhibiting or supplementing expression of any single factor may not be sufficient to efficiently promote remyelination in the complex and poorly understood environment of a demyelinated CNS lesion in MS (Fig. 1.4). Therefore, greater translational understanding is needed to comprehensively address why remyelination fails in disease, and what factors are necessary to develop effective regenerative therapies for MS.

VI. Neural Progenitor Cells (NPCs)

In adulthood one of the major roles of the SVZ is to generate new neural progenitor cells. NPCs are capable of differentiating into neurons, astrocytes, and oligodendrocytes. They have the capacity for self-renewal, migrate extensively throughout the CNS, and when given proper cues can differentiate for tissue repair [119]. Typically, NPCs are identified using markers of pluripotency, such as: Sox2, nestin, and Pax6 [141-143]. This source of potential new cells residing within the adult CNS holds hope for repair in a variety of neurodegenerative diseases including MS. Interestingly, proliferation and mobilization of NPCs from the SVZ has been reported in animal models of seizure, ischemia, brain trauma, and demyelination [118, 144].

The generation of NPCs and their repair capabilities have been investigated in animal models of demyelination [145]. After LPC injection into the rodent corpus callosum the number of NPCs in the SVZ was a significantly increased [117]. Thymidine tracing of these cells revealed that they differentiated into astrocytes and oligodendrocytes in the lesioned area [117]. NPCs derived from the SVZ were also found to proliferate and migrate to lesion areas in the EAE mouse model [116]. Analysis of the SVZ in post-mortem MS cases demonstrated a significantly increased NPC population compared to age-matched control tissue [118]. In addition to increased proliferation, it has also been demonstrated that human NPCs have the capability to migrate to

chronic inactive lesioned areas, yet they are somehow inhibited from differentiating into myelinating oligodendrocytes [118]. These human data suggest that endogenous human NPCs in MS patients have the capability to proliferate and migrate to lesion areas, yet cues from the diseased microenvironment could be impeding differentiation, or that there may be inherent differences in the potential of NPCs in MS that contribute to poor remyelination in this disease (Fig. 1.4).

Another way to test NPCs in demyelinating rodent models is by transplanting them and then assaying the effect they have on myelin injury and repair. This approach has been successfully applied to demonstrate that murine NPCs when grafted into mouse models of inflammatory demyelination have been shown to be potently anti-inflammatory and neuroprotective [146-148]. In a recent paper from the Pluchino group NPCs transplanted into mice in the EAE model were found to ameliorate chronic CNS inflammation by reducing succinate levels acting as free radical scavengers [149]. NPCs, when maintained in an undifferentiated state and are then grafted into an EAE model have been found to have the capability of inducing apoptosis in activated T cells that enter the CNS thereby helping to protect against myelin damage [146]. Additionally, NPCs when grafted into demyelinated lesions have been found to promote the differentiation of endogenous OPCs, likely through the secretion of pro-differentiation factors such as leukemia inhibitory factor (LIF) [150-155]. Previous data from our lab has also demonstrated that human embryonic stem (ES) cell-derived NPCs were capable of attenuating demyelination in the cuprizone model following intravenous administration [156]. The effect of the human ES NPCs in this model were found to be neuroprotective and not limited to cell replacement, consistent with the notion that a function of NPCs is to maintain and/or restore homeostatic processes. These data all suggest that human NPCs can be studied effectively using murine models, and that endogenous NPCs in the MS brain are likely active participants in CNS remyelination.

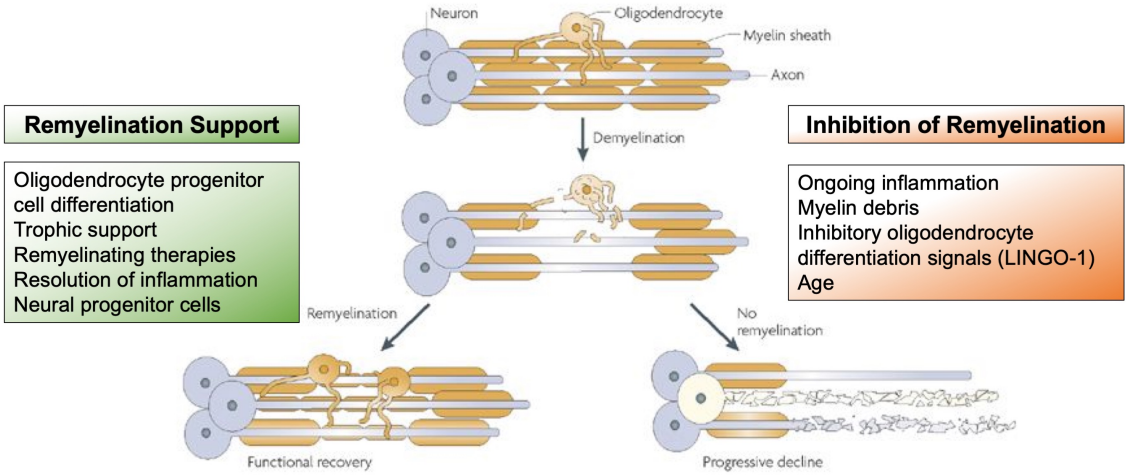

Figure 1.4. Remyelination support and inhibition. After demyelination in the CNS a demyelinated axon can be remyelinated or remyelination fails, which leaves the axons vulnerable to degeneration, associated with the progressive clinical decline in MS. Remyelination occurs when OPCs differentiate and wrap demyelinated axons. OPC differentiation can be enhanced and promoted by positive factors in the lesion area, remyelinating therapies, the clearance of inflammation, and the presence of neural progenitor cells that can be anti-inflammatory, secrete factors to promote OPC differentiation, and differentiate themselves into myelinating oligodendrocytes. Remyelination is inhibited by inflammation, the presence of myelin debris, inhibitory signals, and age. Figure adapted from Franklin and Ffrench-Constant, 2008 [83].

Lastly, through the technology of induced pluripotent stem cells (iPSCs), NPCs from patients with MS can be tested in these mouse models to determine if there are inherent defects. Previous work has demonstrated that OPCs differentiated from iPSCs, derived from patients with primary progressive MS, are equally capable of maturing into myelin-forming oligodendrocytes just as well as age-matched, non-diseased individuals when provided the correct cues *in vitro* [157]. On the other hand, this thesis will further delve into the application of iPS-derived NPCs from PPMS patients which I have now determined are less able to provide neuroprotection to myelin injury and also fail to support OPC differentiation *in vitro* [111].

VII. Emerging Remyelinating Therapies

All current disease modifying therapies on the market for treating MS patients only target the immune system, and none were developed with the primary mode of action to specifically promote remyelination or myelin repair. Considerable research effort has now been focused on promoting remyelination and providing neuroprotection in the brain in order to slow progression, especially in PPMS and SPMS, where there are very little to no effective treatments. The first example of this new approach to treating MS is an experimental antibody drug, anti-LINGO-1 (also known as opicinumab), which blocks LINGO-1 to promote the differentiation of OPCs. LINGO-1 is selectively expressed on OPCs and neurons, where it is associated with the Nogo-66 receptor complex that when activated inhibits neurite growth and oligodendrocyte differentiation [158, 159]. Treatment of mice with the anti-LINGO-1 antibody after focal demyelination in the spinal cord using lysolecithin was found to promote faster remyelination and this new myelin had increased thickness [160]. Anti-LINGO-1 has also been found to reduce axon degeneration and promote regeneration of axons in the EAE model [161]. Unfortunately anti-LINGO-1 failed in Phase II trial in patients with MS, where it failed to reach its primary endpoint: an integrated measure of MS progression and disability markers [162]. While there was no measurable benefit seen in the primary study endpoints, the trial did identify increased percentage of improvement

responders at specific doses compared to placebo, which could help identify sub-populations of MS patients which would respond well to this drug. The primary indicators of anti-LINGO-1 benefit in MS patients included younger individuals who had a shorter disease duration [162].

In the last couple of years new technologies have been developed to be able to screen for remyelinating therapeutics much faster using automated computer programs. Specifically, the Chan lab at UCSF developed an approach to quantify the myelinating potential of OPCs in a high throughput manner they call binary indicant for myelination using micropillar arrays (BIMA). This approach was developed and validated as a rapid assay to evaluate the effects of compounds that can promote OPC differentiation. In BIMA, OPCs are plated at the base of micropillars and through confocal imaging concentric wrapping of myelin, seen as rings, can be imaged and quantified [110]. By using this method over 1,000 FDA-approved bioactive molecules were screened, and a cluster of anti-muscarinic compounds were identified to promote OPC differentiation. Using this high-throughput method clemastine and benztropine were identified to enhance OPC differentiation *in vitro*. They are both FDA-approved compounds that readily cross the BBB, and were found to promote remyelination *in vivo* in the cuprizone, EAE, and lysolectithin models [110]. Another paper, using a high-throughput method to assay drugs to promote OPC differentiation also uncovered benztropine as a potent mediator of differentiation, as well as clobetasol and miconazole [163]. These drugs were also shown to enhance maturation of OPCs *in vivo* in the EAE and lysolectithin models and *in vitro* using human OPCs [163]. Lastly, benztropine was also discovered to be beneficial in promoting remyelination in a paper published by Deshmukh et al. where another high-throughput assay identified this compound. Again, it was shown to decrease clinical severity seen in EAE and help facilitate quicker remyelination in the cuprizone model [164]. All of these studies provide promise for development of remyelinating therapies for MS, especially for patients with progressive MS. Additionally, all of the studies mentioned above used a library of FDA-approved drugs, including compounds known to cross

the BBB, therefore the safety profiles of these tested agents are already known, expediting clinical development.

Clemastine, an FDA-approved antihistamine, was tested in a Phase II trial in patients with RRMS along with a chronic demyelinating optic neuropathy [165]. Patients were specifically selected with this optic neuropathy, along with RRMS, since the latency delay can be easily measured using visual-evoked potentials, and the delay is highly associated with the demyelination of the optic nerve [166, 167]. The shorter the delay in transmission, the more remyelination that has occurred along the optic nerve. Patients treated with clemastine had a reduced latency delay by 1.7 ms/eye, making this the first study that demonstrates the use of a drug with a remyelinating effect in humans that has no known immunomodulatory effects [168]. The use of high-throughput assays in testing potential remyelinating therapeutics led to the discovery of a drug that induced repair in a chronic neurodegenerative condition. A combinatorial therapeutic decreasing inflammation and promoting OPC differentiation may be an effective way to manage and delay MS progression.

Chapter 2: Stem Cells and Disease Modeling

I. History of Stem Cells

Stem cells are defined by their innate potential for regeneration, or self-renewal, where they can divide to produce more of the same type of stem cell, or differentiate into a mature cell type. Self-renewal is defined as the ability for the cell to go through numerous rounds of proliferation while maintaining an undifferentiated state. Stem cells come in different forms. Each type of stem cell has a varying potency, which is the capacity to differentiate into a specialized cell type. Totipotent stem cells can differentiate and construct a complete and viable organism formed during sexual and asexual reproduction, such as a zygote [169]. Pluripotent cells can differentiate into cells of any of the three germ layers: endoderm, mesoderm, or ectoderm. Multipotent cells can differentiate into only specific cell types. One example of particular interest to multiple sclerosis, also the focus of this thesis, is neural progenitor cells, which can differentiate into oligodendrocytes, astrocytes, or neurons.

The first observations of stem cells were in the 1960s and defined as hematopoietic stem cells (HSCs). These stem cells give rise to blood cells, including leukocytes, erythrocytes, and thrombocytes [170]. HSCs undergo constant self-renewal and can differentiate into multiple mature cell types. Since the discovery of HSCs they have been used for transplantation procedures in patients with cancers of the blood or bone marrow, where the immune system is destroyed and replaced with new HSCs. HSC transplants (HSCT) have been found to ameliorate both acute and chronic forms of EAE, through immunosuppression of activated T cells and B cells [171, 172]. Like many stem cell studies have found, the impact of stem cells on disease processes are manifold: HSCs have been found to suppress CNS inflammation through the secretion of soluble factors, cell-contact, and indirectly through actions on other cell types to inhibit T cell activation [171, 173, 174]. Based on these positive results in animal studies of MS, HSCT are now being conducted in human RRMS patients using their autologous bone marrow in order to avoid transplant rejection. To date, even though it is a rough procedure that involves intense

immunosuppression, HSCT has been reported to be well tolerated by patients [175]. In an initial phase I/II trial, MS patients receiving autologous HSCT did not experience any significant side effects or infections, but only a small number of patients felt any benefit from the transplantation [176]. An additional HSCT trial using patients with progressive MS also demonstrated that autologous HSCT could stabilize lesions after one year post-transplant, although this was reported in only half of the patients treated [177]. Overall, the assessment of HSCT as a treatment for MS is exploratory and subject to ongoing study. It has been shown to be safe in patients with MS, but the overall benefits are at this point limited to a small subset of patients tested and the utility of this approach to the wider patient population has yet to be determined.

Embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass of a blastocyst. ESCs have the ability to differentiate into the three primary germ layers, making them ideal candidates for tissue engineering [178]. For the first time in the 1990s scientists were able to generate all of the different cell types of the human body in a dish [179]. These groundbreaking studies offered the possibility of using these differentiated cell types to test therapeutics, study human tissue regeneration, and/or develop expansive arrays of cells for transplantation. Yet, these cells were developed from human embryos which represented a significant ethical hurdle for research and mass production. From the perspective of patients with MS or Parkinson's disease, human (h)ESCs offered a way to regenerate new cells to "fix" their brains. Whether functional OPCs to recover myelin, or new dopaminergic neurons that could be grafted into the brain, the promise of hESCs was widely heralded as a cure for many diseases [179]. The first human ESC lines available were originally generated for *in vitro* fertilization (IVF) but were then never used clinically. With consent from the donors the blastocyst was taken to generate human ESC lines [180]. Initially, about 20 lines were generated and isolated prior to August 2001, when the United States government started to restrict human ESC research due to the ethical concerns about the source tissues. At that time, the field had already generated ESC lines that the NIH had approved which became the benchmark human lines for research

purposes. The small number of hESC lines represented a major impediment to the purported future application of ES cell-based therapies. Their use as a clinical reagent was limited as all human ESC lines that had been developed were isolated in the presence of animal proteins and growth factors which limits their potential for transplant due to immune-rejection [181].

The two best examples of successful application of human ESCs in rodent models involve the transplant of a differentiated cell type. Transplantation of human ESC-derived dopaminergic neurons into a mouse model of Parkinson's disease successfully reversed behavioral defects and grafted long-term in the striatum [182, 183]. Oligodendrocytes, derived from human ESCs, were also shown to promote remyelination in animal models of demyelination, and also repair white matter lesions after spinal cord injury [184]. Transplantation of NPCs derived from a human ESC line were also found to be neuroprotective in the cuprizone mouse model of demyelination [156]. Despite these promising results in mouse models of CNS demyelination, translation of ESCs into human clinical disease brings with it significant risk, including the caveats associated with contamination of these cell lines with animal products which can elicit potent immune reactions in humans, immune rejection due to genetic incompatibility of the host with the ESC line, the potential for tumor formation if any of the transplanted cells are left undifferentiated, and the ethical concerns in derivation of the cells [181].

In 2006, a significant development in stem cell research transformed the field. This discovery was of genes that can induce pluripotency in somatic cells. First published in 2006, a research group at Kyoto University led by Shinya Yamanaka, found that murine somatic cells, from adult mice, could revert to an embryonic stem cell-like state by transducing the cells with transcription factors Oct4, Sox2, c-Myc, and Klf4 [185]. These induced pluripotent stem cells (iPSCs) were found to express the same cellular markers of ESCs, differentiate into a variety of cell types like ESCs could and therefore offered potential to make ES-like cells without the ethical quandaries surrounding collection of human embryonic tissues [185]. In the following year, two groups reported that they were able to generate iPSCs from adult human somatic cells using the

same approach, but also using a new combination of transcription factors: Oct4, Sox2, NANOG, and Lin28 [186, 187]. Just like the murine cells, the human iPSCs were found to generate all three germ layers [178, 186]. Based on this research, the Nobel Prize for physiology and medicine in 2012 was awarded to Shinya Yamanaka and John Gurdon "for the discovery that mature cells can be reprogrammed to be pluripotent" [188]. For the first time, adult somatic cells had the possibility to be reprogrammed into an embryonic-like state, without the controversial practice of using human ESCs, which has led to a torrent of research using these iPS cells to model human disease. With this new approach, disease-specific iPS cells can be used to model the human condition, test therapeutics, and generate a source of cells to study disease, a potentially unlimited source of transplantable cells, among other possible utilizations.

II. The Use of iPS Cells in Research

After the landmark discovery of iPSCs great effort has been made to generate the various cells of the human body for purposes such as interrogating the genetics of specific cells, performing drug screening assays, and even using them as a cell replacement in humans (Fig. 2.1). For the purpose of studying multiple sclerosis, protocols for the differentiation of both murine and human iPSCs into oligodendrocytes has been established over the last couple of years, and have gone from a 3 month protocol to only 28 days [189, 190]. Recently, a protocol for the generation of human cortical spheroids, with mature, myelinating oligodendrocytes was published, demonstrating their use in testing compounds such as clemastine and ketoconazole in promoting myelination [191]. Using iPSCs from patients with Pelizaeus-Merzbacher disease (PMD), a genetic leukodystrophy affecting myelin, the human cortical spheroids recapitulated the defect in myelination and allowed for screening of various treatments [191]. Interestingly, only oligodendrocytes derived from patients with a specific genetic myelin disorder, such as PMD, have been found to show deficits [191]. On the other hand, myelinating oligodendrocytes differentiated *in vitro*, with the proper cues, can be generated from iPSC lines developed from

patients with PPMS, demonstrating that there is no one genetic defect impairing these cells in PPMS, and the defect in patients may be due to epigenetic changes [157]. This study draws into question exactly what happens during the reprogramming stage of iPSCs, and if unique epigenetic changes are maintained, and exactly what genetic information is retained related to cell type, which may be integral to understanding why OPCs fail to remyelinate in progressive MS.

A large amount of studies have shown that there is interplay between environmental factors and individual genetic susceptibility, which is likely to produce a predisposition to MS. It is from this standpoint that understanding epigenetics, and epigenetic changes in specific cell types is considered crucial to uncovering and developing therapeutics to treat MS patients [192]. Epigenetic modifications are changes that affect gene expression, without the alteration of the DNA sequence, which includes DNA methylation and histone modification [193]. In this respect, some studies that have suggested that cellular reprogramming of somatic cells can alter genomic methylation and epigenetic modifications may be cause for concern for using iPS cells to model non-genetic disease conditions. However, numerous studies have now demonstrated that even with reprogramming cells retain forms of “epigenetic memory”, including in some cases memory of the somatic tissue of origin, which suggests that the notion of reprogramming results in "resetting" epigenetics or wipes clean epigenetic markers associated with disease is simply untrue [194, 195]. For example, murine keratinocytes reprogrammed into iPSCs differentiate more readily into fibroblasts, demonstrating a maintained memory through the reprogramming process [196]. Analysis of DNA methylation in donor-specific iPSC lines found that these epigenetic modifications were unchanged during the reprogramming process from the somatic cell [197]. iPSCs have also been used to model nonfamilial, sporadic forms of disease, such as Alzheimer’s. Neurons differentiated from iPSCs from patients with genetic, familial Alzheimer’s as well as sporadic Alzheimer’s exhibited higher levels of amyloid-β and phospho-tau [198]. In addition, age-related mitochondrial DNA mutations were found in iPSCs derived from fibroblasts of aged

individuals, compared to fibroblasts from young subjects, demonstrating that reprogramming of cells does not completely wipe clean epigenetic markers [195]. Using iPSCs as disease models reflects individual epigenetic changes unique to each patient or disease state, based on the somatic tissue of origin, but differentiation of iPSCs can modify some of these original methylation markers [194]. It is important to point out that human iPSCs are known to retain epigenetic markers from the patient somatic cell when reprogrammed and differentiated into a cell type of interest, but the myriad factors that potentially influence this are as varied as the individual human donors. Studies to date have reported that epigenetic differences can depend on exactly how the cells were reprogrammed, the cell culture conditions used, the initial somatic cell used for reprogramming, the genetics of the donor patient, and cell types that that iPS cell line is then differentiated into [194]. In spite of these technical caveats, over the past decade iPSCs have made it possible to model "human diseases in a dish" [199]. Although these cells offer the potential for novel insights into these enigmatic diseases, our limited understanding as to the exact epigenetic changes that can occur with disease or during reprogramming and differentiation need to also be considered.

Due to the inaccessibility of primary tissues, such as brain, and the limited accuracy of animal models for the study of multiple sclerosis, patient-specific iPSCs represent a potentially powerful tool to understand the mechanisms underlying this disease. Diseases with full genetic penetrance, such as Huntington, display more robust cell-autonomous defects [194], but since MS is a complex disorder not caused by any single genetic mutation, it has been much more difficult to parse apart possible cellular defects that contribute to the disease phenotype clinically defined as MS. One of the main goals of this thesis has been to use iPS cells from MS patients to address this important question.

Several studies have successfully generated iPSC lines from MS patients with varying forms of disease. These studies have also used these cells to generate various differentiated cell types [111, 157, 200]. In particular, the generation of oligodendrocyte progenitor cells offered the

potential to understand inherent limits on remyelination in MS. A first study generated iPSCs from patients with RRMS reported that they were successfully able to differentiate iPS cells into mature astrocytes, oligodendrocytes, and neurons and these neurons exhibited electrophysiological characteristics no different from differentiated cells from control iPS cell lines [201]. Using a different protocol, Douvaras et al. demonstrated that OPCs could be generated from iPSC lines derived from patients with PPMS. These OPCs were differentiated *in vitro* both efficiently and with comparable competence to controls when provided the proper growth factors. These cells were also found to be functionally viable to provide *in vivo* myelination when transplanted into the forebrain of neonatal shiverer mice, a mouse line that lacks myelin basic protein and thus native mature myelin [157]. Thus, these early studies suggested that iPS cells from MS patients when differentiated into astrocytes, oligodendrocytes, or neurons were phenotypically "normal".

Experimental evidence with rodent neural progenitor cells has demonstrated that when these cells are transplanted into mouse models of demyelination they can provide neuroprotection and promote differentiation of endogenous OPCs, likely through the secretion of growth factors [150-152, 155]. More recently, human NPCs have also been found to attenuate inflammation and promote CNS repair in mouse models, [111, 151, 202-204]. Yet, my more recent work has demonstrated that NPCs derived from PPMS patients' iPS cells lack neuroprotective properties in a mouse model of MS. Based on the data presented in this thesis more needs to be understood about NPCs from patient iPSCs with MS before they could be used or autologous stem cell transplants into patients.

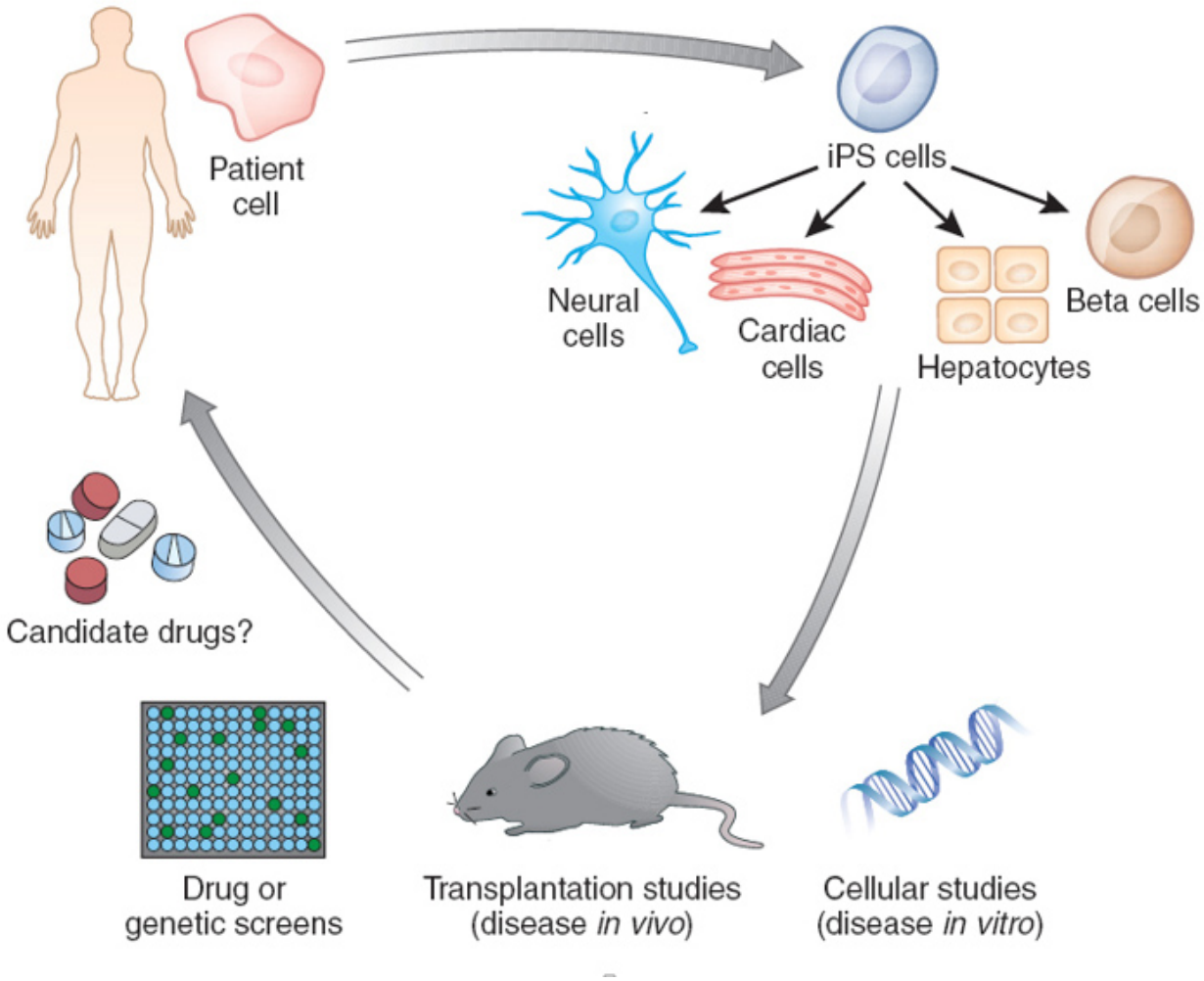

Figure 2.1. Patient-derived cells are reprogrammed to iPSCs and differentiated towards specific cell types to allow for analysis *in vitro* and well as *in vivo*. Figure adapted from Memorial Sloan Kettering Cancer Center.*

* https://www.mskcc.org/sites/default/files/node/35635/images/disesae-modeling.jpg

III. Utility of iPS Cells in Disease-Based Drug Screens

Patient-specific human cells developed from iPSC lines have also been used in order to test possible therapeutics (Fig. 2.1). In the context of MS, drugs that promote OPC differentiation into functional, myelinating oligodendrocytes have been tested on patient cells, as well as control cells, which has led to the discovery of clobetasol and miconazole as promoters of human oligodendrocyte differentiation [111, 163]. When human OPCs, derived from patients with MS and controls, are given the proper growth factors and cues to differentiate they can myelinate properly [157, 205]. The fact that OPCs from patients with MS can be readily generated, and function normally *in vivo*, holds promise as a potential cell replacement strategy.

More recently, a new model to study human-iPSC oligodendrocytes called “oligocortical spheroids” has been developed. This 3D culture system was found to recapitulate cell-cell interactions, microenvironments, and could be used to extend the temporal dynamics of cellular interactions *in vitro* more accurately than a traditional 2D cell culture system [191]. This approach was demonstrated using iPSCs developed from patients with Pelizaeus-Merzbacher disease (PMD), an X-linked disease associated with defects in myelin production. The oligocortical spheroids allowed researchers to understand the developmental errors occurring in developmental myelination and were able to correct it with targeted gene therapy and test remyelination therapies, such as clemastine [191].

Overall, the ability to generate patient specific iPSCs that can self-renew and then differentiate into essentially all somatic cell types gives them immense potential for a variety of applications. Some include using them to model multitudes of diseases and perform pharmaceutical testing and gene targeted therapies, test them in animal models, and analyze gene changes in varying cell types among other uses. There are still some challenges that remain and need to be addressed before autologous human cell transplants can occur. Improvements in differentiation methodologies, and new technologies, such as the 3D cultures, allows for better models and platforms that can be combined with traditional drug development to link together

genetic patient risk as well as cellular and molecular events that can lead to disease onset and progression. Studying MS using patient-specific iPSCs has the potential to reveal epigenetic trends within patients, test adult stem cells for the potential to promote brain regeneration, and help understand why progression occurs.

The objectives of this project were to generate neural progenitor cells from patients with primary progressive multiple sclerosis in order to determine if there were inherent defects in their functioning. I discovered an intrinsic difference in iPS cell-derived NPCs from PPMS patients compared to age-matched controls, where they failed to promote endogenous remyelination due to a senescent phenotype. This data demonstrates a fundamental difference in the disease state, which reflects a central role in NPCs modulating the MS lesion environment.

Chapter 3: Aging and the Central Nervous System

Aging is the main risk factor for many chronic neurodegenerative diseases, including Alzheimer's disease and Parkinson's, as well as diabetes, arthritis, and cardiovascular disease [206]. Overall, aging is associated with a progressive loss in the ability of tissues to recover from damage and increased tissue vulnerability to stress [207]. Due to the rise in average life expectancy over the past decade, it is estimated that by 2050 two billion people will be over 60 years of age, drastically increasing the prevalence of chronic diseases [208]**.** Therefore, it is crucial to develop interventions that can promote better tissue regenerative capacity with aging that could also extend healthy life span (also referred to as "healthspan").

Recent advances in the field of molecular aging have determined that senescent cells accumulate with age and disease in various tissues and these cells are believed to be the driving force of tissue aging [209, 210]. Cellular senescence is defined as a chronic stress response. It can be induced by damage, sometimes associated with growth arrest, such as UV irradiation, and this cellular phenotype is often accompanied by a senescence-associated secretory phenotype (SASP) [209, 211, 212]. The SASP is generally a collection of pro-inflammatory and matrix-degrading molecules that are released from senescent cells. It is the result of the SASP factors that the function of surrounding cells are affected. Most especially, tissue resident stem cells are known to be impaired by the SASP which is thought to underscore the limited regeneration of tissues with increased age [213]. This chapter will focus on describing cellular senescence and a role for cellular senescence in multiple sclerosis, with the possibility that this process may contribute to failure of myelin regeneration in this disease.

I. Cellular Senescence

The first discovery of replicative senescence *in vitro* came from Hayflick and Moorehead, who demonstrated that normal human fibroblasts exhibited a limited potential for replication with time [214]. This observation led to the idea that replicative senescence contributes to aging, where

the progressive depletion of cells limits that tissue's potential to replicate, repair, or regenerate once damaged [214]. Since these observations of cellular aging are referred to as “replicative senescence”, cell cycle arrest has become a model to study aging *in vitro*. In 1998, it was discovered that the attrition of telomeres, the repeating nucleotide sequences at the ends of chromosomes, also underlies replicative senescence [215]. The shortening of telomeres triggers a DNA damage response (DDR), which recruits the damage sensor ataxia telangiectasia mutated (ATM) to stabilize and upregulate expression of proteins p53 and p21 [216]. p21 then prevents cyclin-dependent kinase 2 (CDK2) from inactivating the protein retinoblastoma (Rb), which then inhibits that cell from entering S phase and thus blocks replication [217]. Another pathway overactivated in cellular senescence is the mammalian target of rapamycin (mTOR) pathway. In senescent cells, mTOR activity can lead to an upregulation of $p16^{Ink4a}$ which prevents CDK4- and CDK6-mediated inaction of Rb, which, in turn, blocks the cell cycle [217]. Both of these mechanisms can occur due to a multitude of stressors such as reactive oxygen species (ROS), mitogenic/oncogenic signaling, stalled DNA replication, chromatin disruption, and other DNA-damaging stressors such as ultraviolet (UV) or gamma irradiation, and chemotherapeutics [218-220].

It is important to note that there is increasing awareness that there may be a variety of forms of cellular senescence and the specific form of cellular senescence may depend upon how this process is initiated. For example, there are differences in expressed proteins and genes between stress-induced senescence and replicative senescence [221-223]. Replicative senescence causes a total arrest of the cell cycle, while cells with stress-induced senescence maintain replicative competence although still express molecular hallmarks of senescence, including production of pro-inflammatory SASP factors [223]. Stress-induced senescence is due to a gradual and cumulative burden of oxidative damage which occurs with aging, and can lead to cell cycle arrest once a theoretical threshold is reached, although cell cycle arrest is not a prerequisite for stress-induced senescence [216].

$p16^{Ink4a}$ upregulation is the most common marker used to identify senescent cells. $p16^{Ink4a}$ has been used as a biomarker of natural aging as its expression is known to increase with time in various tissues [224, 225]. Studies using the $BubR1^{H/H}$ mouse, a model of rapid aging, have demonstrated that tissues in these mice exhibit premature aging and increased $p16^{Ink4a}$ expression [226]. BubR1 is a mitotic checkpoint protein, and the low levels in the mouse model ($BubR1^{H/H}$), due to two hypomorphic alleles, causes premature separation of sister chromosomes inducing aneuploidy and progeriod phenotypes. Recent studies using mice have demonstrated that targeted ablation of $p16^{Ink4a}$ positive cells can be used as a strategy to study cellular senescence. One of these models is termed the INK-ATTAC, which selectively expresses a drug-inducible FKBP-caspase-8 fusion molecule under control of the $p16^{Ink4a}$ promoter, which then initiates apoptosis when a specific drug is administered to the mice [227]. Induced deletion of $p16^{Ink4a}$ from senescent cells has been shown to extend lifespan and reverse age-related functional decline in organs such as kidneys and pancreas [227, 228]. Similarly, targeting of cellular senescence by deletion of $p16^{Ink4a}$ positive cells has also been found to mitigate accelerated aging in diseases such as progeria modeled in the $BubR1^{H/H}$ mouse, and more recently in glia in a model of Alzheimer's disease (AD); demonstrating $p16^{Ink4a}$ has a central role in the functional effects of cellular senescence [224, 227-232]. These studies have demonstrated that senescent, $p16^{Ink4a}$ expressing cells drive natural aging, as well as age-related diseases, such as atherosclerosis and osteoarthritis [227, 233, 234].

There is no single marker that can be used to universally identify senescent cells, and $p16^{Ink4a}$ is therefore only part of the process of identifying cellular senescence (Figure 3.1). The upregulation and expression of additional proteins such as p53 and p21 are also associated with senescence, but these cellular changes can also occur with conditions that are not senescence. For this reason, it is has become current standard practice for studies in this field to use multiple markers in order to identify and verify cellular senescence [206]. For example, enzymatic activity of the lysosomal hydrolase, identified by β-galactosidase activity at pH 6.0, has become an

established marker of cellular senescence, which is referred to as senescence-associated β-galactosidase assay (SA β-gal) [235]. A practical advantage of this marker is that the colorimetric assay used to detect it works both on *in vitro* and *in vivo* tissues to detect senescent cells [235].

The most distinguishing feature of senescent cells is the production of a SASP (Table 3.1). This aspect of altered cellular function functionally and biochemically distinguishes cellular senescence from cellular quiescence. While the specific factors that contribute to the SASP can vary depending on cell type, in general, there are several factors consistently produced by senescent cells, including those associated with oxidation and inflammation, including interleukin (IL)-6, IL-1, MIP1β, MIP1α, MMP-3, HMGB1 as well as growth factors such as FGF2 (Table 3.1) [212, 236, 237]. With progressing age, many of these SASP factors have been found to be increased on an organism-wide scale, especially IL-6 [238]. Because of the secretion of pro-inflammatory SASP factors from senescent cells, only a relatively small number of senescent cells are necessary to evoke physiological changes and alter function of a tissue [212]. Chronic expression of the SASP impairs the proper function of neighboring cells, and is therefore thought to be a major contributor to inflammaging [239]. Inflammaging is a theory that low, but chronic, levels of inflammation drives age-related decline in function, which is supported by the presence of the SASP [211, 239]. The SASP phenotype is also notable for its absence of several factors namely, IFNγ, NGF, and TIMP-1. In fibroblasts, for instance, many factors known to be involved in leukocyte recruitment are also reduced, including CX3CL1, PDGF-BB, and anti-inflammatory cytokines, such as IL-10 and IL-12 are also not expressed by senescent cells [240]. SASP expression is variable between different types of senescence, especially depending on the initial causes of senescence in that particular cell population [213, 237, 241]. In addition, the secretome of the SASP is highly variable from cell type to cell type, and varies based on how long the cell has been senescing, and therefore impacts surrounding cells differently depending on the tissue [212, 213].

Factors which comprise the SASP have been found to promote tumor development in breast and prostate cancers through the secretion of growth factors, such as GROα and CTGF, [242, 243]. These data point to an important contributing role for the SASP in senescence-related diseases, which, relevant to this thesis, includes the influence of the SASP on tissue resident stem cells and impaired tissue regeneration. Chronic exposure of the SASP factor IL-1 is known to inhibit the proper differentiation of hematopoietic stem cells thereby preventing their homeostatic roles [244]. Similarly, IL-6 is required for both the induction and continued maintenance of pluripotency in stem cells, therefore secretion of IL-6 by senescent cells is thought to sustain stem cells in an undifferentiated state and compromise the ability of these cells to mediate tissue repair [245]. Senescence has also been shown to trigger cell "reprogramming" *in vivo*, promoting an accumulation of undifferentiated stem cells prevented from differentiating [246]. de Keizer has termed this phenomenon the ‘senescence-stem lock’ model, where SASP factors keep their neighboring cells locked in a stem-like state, which in the context of aging and diseases of injury, would prevent tissue rejuvenation or repair [247]. While these findings point to an important function of the SASP as a means by which cellular senescence can impact tissues and organ function, our current knowledge of the SASP, and how it varies across different cell types, remains quite limited. Future studies to understand how the SASP is generated, and why specific factors are being secreted in senescent states may help improve our understanding on why tissue regeneration fails with age and in specific diseases.

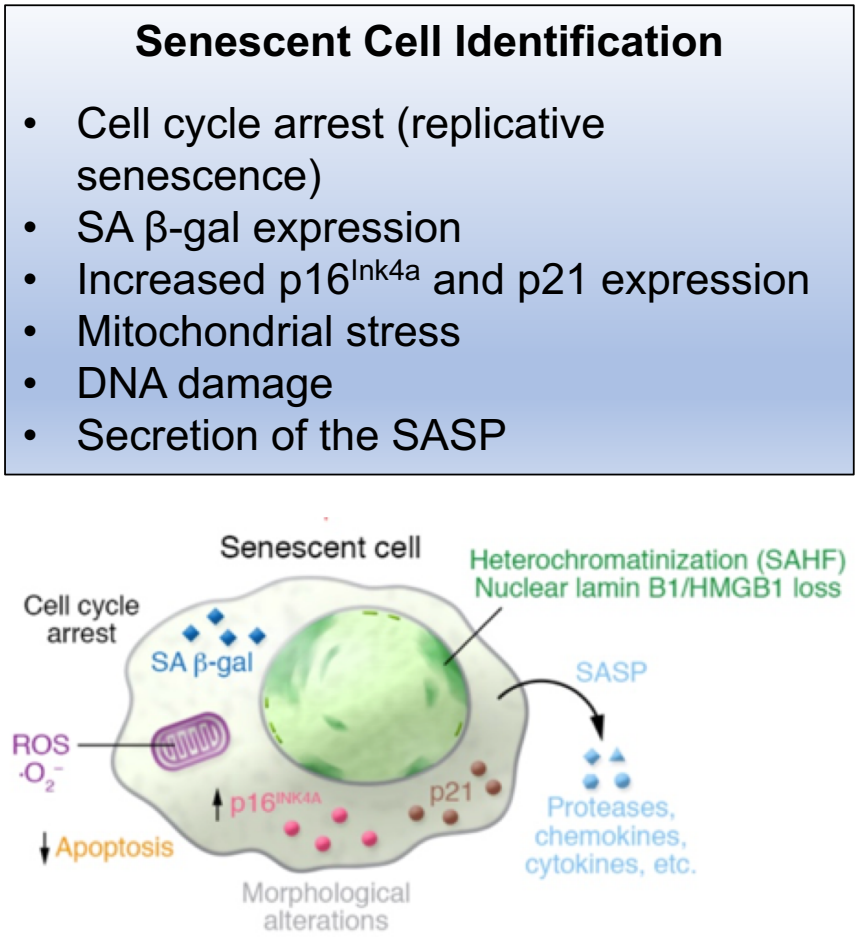

Figure 3.1. Identification of senescent cells. Senescence can be identified by markers indicated above. Since senescence is unique depending on the cause as well as the cell type, not all senescent cells express each hallmark consistently. Figure adapted from Baker and Petersen, 2018 [206].

II. Cellular Senescence in the CNS

Cellular senescence has been identified in multiple CNS cell types in aging and models of neurodegenerative diseases. Recent studies have identified hallmarks of cellular senescence in astrocytes and microglia in models of Alzheimer's disease (AD) and Parkinson's disease (PD) [229, 248-250]. The chronic low-grade inflammation that occurs in the brain with age, has been shown to contribute to the decrease in the numbers of synapses and dendritic spines on neurons, both integral to proper neuronal function, which has been suggested to underlie cognitive impairment and memory loss with age [251, 252]. While neurons of aged mice are known to accumulate high amounts of DNA damage and express pro-inflammatory molecules, which are also hallmarks of senescence [218, 253], it has not yet to be determined whether neurons themselves become senescent, or if they are subject to senescence from other cell types, and whether these changes contribute to the decline in CNS function with age. With age, the human brain has been found to develop increased markers of cellular senescence. Human brain tissues exhibited increased $p16^{Ink4a}$ positive astrocytes, along with increased levels of MMP-3. While these changes are suggestive of cellular senescence, it is currently not known exactly how aging drives major features of declining brain function in aging or in neurological disorders such as losses in cognitive function, [229].

Microglia are a cell type in which cellular senescence has been identified. In aged mice, microglia have been shown to exhibit shorter telomeres and up-regulate the production of pro-inflammatory cytokines, including: IL-6, IL-1β, and TNF-α [254, 255]. When microglia are kept regularly activated *in vitro* they too express hallmarks of senescence including SA β-gal activity, heterochromatic foci formation, and growth arrest [256]. More recently, using a transgenic model to delete $p16^{Ink4a}$+ senescent cells (called the INK-ATTAC mouse model) when crossed with an AD tau-dependent mouse ($MAPT^{P301S}$PS19) it was reported that specific loss of senescent microglia and astrocytes preserved cognitive function and prevented gliosis [231]. These results show that senescent glia have a prominent role in the progression of tau-mediated AD [231].

Cellular senescence has also been reported in astrocytes. In response to reactive oxygen species (ROS) exposure astrocytes exhibit markers of senescence, including growth arrest, SA β-gal activity, and increased $p16^{Ink4a}$ and p21 expression [257]. Exposure to the herbicide paraquat, an herbicide associated with an increased risk of idiopathic Parkinson's, has been shown to induce senescence in astrocytes which contributes to dopaminergic neurodegeneration [249]. In addition, histological analyses of brain tissues from Parkinson's patients identified senescent markers in astrocytes [249]. Therefore, in PD it is thought that the chronic cellular stress linked to this disease may evoke cellular senescence in astrocytes that leads to the manifestation of disease [206]. Importantly, astrocyte dysfunction, and markers of stress-related oxidative stress in astrocytes are found in many neurodegenerative diseases, suggesting that cellular senescence in astrocytes may be a component of many neurological conditions. In support of this point, current data indicates that cellular senescence can be a pathological gain-of-function adaptation in chronic neurodegenerative diseases.

Aging researchers have begun to examine how cellular senescence contributes to other neurological diseases, such as HIV-associated neurocognitive disorders (HAND). While in the 1990s an HIV diagnosis was tantamount to an early death, the advent of anti-retroviral drug therapies have enabled patients to control the levels of HIV. However, longitudinal studies are now finding that HIV patients are prone to developing complications associated with premature aging, such as HAND, that are believed to be related to highly active antiretroviral therapies themselves [258]. Hence, the treatments for HIV may be initiating cellular senescence and an aged phenotype in this patient population. Recent studies looking to cellular senescence have found that treating astrocytes *in vitro* with antiretroviral drugs can induce markers of cellular senescence, as well as increased ROS production [259, 260]. Microglia are also known to develop characteristics of cellular senescence after exposure to HIV itself, but this process is not yet well characterized [261]. Based on these findings, our limited understanding of how senescent cells contribute to neurodegeneration in disease warrants greater investigation.

Table 3.1. SASP Factors. Adapted from Coppé et al., 2010 [212].

Increased Factors in the SASP		
IL-1, -6, -7, -8, -13, -15	MIP1β	FGF
IL-1a, -b	IGFBP-2, -3, -4, -6, -7	HMGB1
MIP1α	MMP-1, -3, -10	
MIF	ROS	
VEGF	Fibronectin	
Decreased Factors in the SASP		
TIMP-1	CX3CL1	PDGF-BB
IL-10, -12		

III. Inhibition of Senescence

The INK-ATTAC mouse has provided compelling evidence that specific removal of senescent cells can be beneficial in modulating the impact of inflammatory diseases and aging. This approach has opened up a novel field of therapeutic pharmacology which seeks to specifically eliminate senescent cells as a treatment. Since mice globally lacking $p16^{Ink4a}$ are prone to developing tumors, another transgenic model termed p16-3MR was developed [262]. These mice express a trimodal reporter construct under the control of the entire $p16^{Ink4a}$ promoter, that is coupled to a red fluorescent protein, synthetic Renilla luciferase, and a truncated herpes simplex virus thymidine kinase (HSV-TK) [263]. When ganciclovir is given to the mice HSV-TK converts it into a toxic compound, triggering apoptosis in $p16^{Ink4a}$ -expressing cells [263]. This mouse model has helped to understand, and identify, exactly what cells become senescent in a variety of age-associated diseases [233, 234]. Since this mouse model is not widely available, current methods to identify cellular senescence rely on expression of markers, in particular $p16^{Ink4a}$, to identify senescent cells. However, some senescent markers are not unique to senescent cells and can be expressed by other cell types which has potential for complicating the direct elimination of senescent cells and the interpretation of these approaches.

In addition, these transgenic approaches to delete senescent cells are not directly translatable to humans. Because of this, researches have begun to development a new class of drugs called “senolytics”, which pharmacologically target senescent cells for elimination. Many of these developed drugs target the antiapoptotic pathway that is known to be upregulated in senescent cells [264]. Some of these experimental agents include ABT-737 and navitoclax, which prevent the antiapoptotic activity of BCL-2, and thus initiate cell death in senescent cells [265, 266]. More recently, a combination of dasatinib (D), a tyrosine kinase inhibitor, and quercetin (Q), a PI3K pathway inhibitor have been found to be a senolytic combination (D+Q) in animals [267]. In aged mice, and mouse models of disease, D+Q treatment effectively reduced the number of senescent cells in heart, lung, or adipose tissue, while also reducing inflammation [227, 264, 268,

269]. Overall, senolytics have been found to increase health- and lifespan in old mice, yet the overall impact on the CNS are incompletely studied [232].

Rapamycin, an mTOR inhibitor, which was used in my thesis studies, is known to improve health in animal models, inhibit cellular senescence in fibroblasts, and prevent the secretion of the SASP (Figure 3.2) [270-273]. It is established the rapamycin can increase lifespan and inhibit the development of cellular senescence, but the complete mechanism by which rapamycin achieves these effects are also poorly understood [274].

Rapamycin works to block mTOR activity through binding with the FK506-bingind protein, FKBP12. mTOR belongs to a family of kinases made up of two catalytic subunits: mTOR complex 1 (mTORC1) and mTORC2 [275]. mTORC1 suppresses the process of autophagy, which is crucial in generating substrates for energy production. Autophagy has been shown to decline with age, causing an accumulation of damaged proteins and organelles, especially mitochondria, and through inhibition of mTORC1 autophagy can be stimulated [276]. mTORC1 increases mitochondrial functions by stimulating mitochondrial biogenesis and controls the metabolism of cellular energy, increasing glycolytic flux and limiting oxidative phosphorylation [276]. These processes can induce stress in aged cells, and thus lead to senescence, therefore inhibition of mTORC1 by rapamycin in aged cells would be expected to attenuate stress and be beneficial. Inhibition of mTORC1 with treatment of rapamycin was found to enhance intestinal stem cell function by mimicking calorie restriction, allowing them to replicate and differentiate when needed [277]. In addition, a study showed age related decline in the function of hematopoietic stem cell (HSC) function associated with increased activity of mTORC1 [278]. Rapamycin was found to restore self-renewal of HSCs in old mice [278]. Most importantly, rapamycin blunts the pro-inflammatory SASP secreted by senescent cells and extends lifespan and postpones age-associated diseases in mice [274, 279]. Metformin, which also leads to indirect inhibition of mTOR, can increase lifespan and healthspan in mice by mimicking calorie restriction [280].

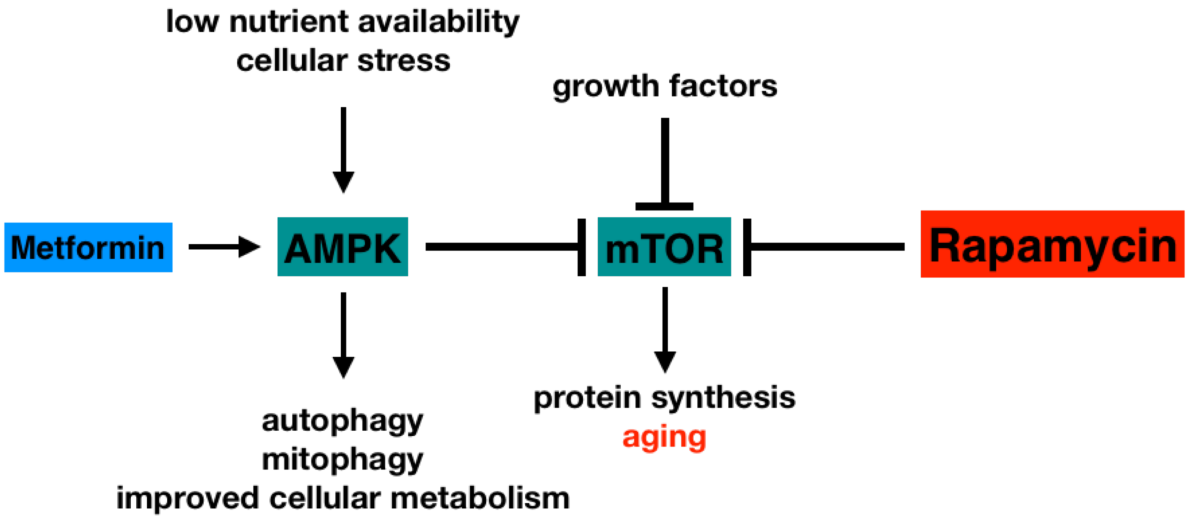

Figure 3.2. Inhibition of cellular senescence using rapamycin. Rapamycin inhibits mTOR which prevents the development of cellular senescence. Metformin mimics benefits seen in calorie restriction by increasing AMPK which improves cellular functioning and inhibits mTOR. Both rapamycin and metformin treatment in mice has been found to increase life and healthspan.

While the exploration of challenging and modifying age-related changes is advancing out the understanding of aging and age-associated changes in disease, some researchers have undertaken studies from quite a different approach. Castellano et al. have been studying how youthfulness can promote rejuvenation and counter the aging-related effects on tissues. They have found that exposure to factors in young blood can counteract age-related changes in the CNS, specifically in the hippocampus of aged mice [281]. Tissue inhibitor of metalloproteinases-2 (TIMP-2), a secreted protein, was found to be enriched in human cord plasma and increase synaptic plasticity and hippocampal-dependent cognition in aged mice [281].

Aging is also known to limit remyelination and aged animals exhibit poor remyelinatng potential. The reasons for this are thought to be due to changes within the lesion microenvironment, which may also relate to epigenetic changes within OPCs themselves that decrease their ability to differentiate and properly myelinate in the milieu of the aged CNS [125, 282, 283]. Interestingly, Franklin and colleagues have shown that remyelination in aged animals can be enhanced using youth: heterochronic parabiosis pairing of an older mouse with a young mouse was found to promote myelin repair in the aged CNS. These seminal findings indicate that factors present in young blood can help rejuvenate repair and these factors, such as TIMP-2, may account for the changes in tissue resilience with age [284].

While these data support aging as a process that can impact CNS myelin, cellular senescence has yet to be identified in oligodendrocytes or neurons, but the awareness of aging in this field is slowly starting to develop. The field of senescence is still in its infancy, but many of the typical hallmarks of senescence have been observed in research involving brain aging, just not termed “senescent”. Overall, based on what we know about senescence, these cells could cause disruptions of the BBB, impaired myelination, and reduced neurogenesis, impairing repair, but much more research needs to be done in order to understand exactly how these cells affect natural aging and human disease [206].

IV. Cellular Senescence in MS

The concept that cellular senescence may contribute to failed remyelination in MS is consistent with the gradual cumulative nature of progressive multiple sclerosis, and that age is one of the most influential factors in the progressive of MS [285, 286]. This concept is a central idea explored in Chapter 5 of this thesis. While we do not know any specific triggers of MS, chronic stress-induced senescence can evoke DNA damage, mitochondrial dysfunction, epigenetic stress, reactive oxygen species, and ER stress - all of these have been implicated in MS [287-291]. In addition, many SASP factors are also known to have elevated expression in MS, including a host of oxidative (e.g. nitric oxide) and pro-inflammatory factors (including interleukin (IL)-6, IL-1, IL-8, MIP1β, MIP1**α**) and growth factors (e.g. FGF2) [264, 292, 293]. As mentioned in the section above, these SASP factors can contribute to disease-related tissue impairment by causing a paracrine spread of cellular dysfunction and foster an environment conducive to tissue damage. These factors can also disrupt the stem cell niche thereby perturbing endogenous spontaneous regeneration of damages or lost tissue [294-296].

There are several lines of evidence to support a role for cellular senescence in experimental models of demyelination. First, it has been recently shown that the cuprizone mouse model of demyelination is associated with induced markers of cellular senescence in glia within demyelinated lesions[†]. Second, middle-aged male mice (8 months) have been reported to have an increased severity of EAE [297] that is associated with senescence in regulatory T cells [298] and increased permeability of the blood brain barrier [299]. Next, senescent CD8+ T cells have been identified in the aging human brain, and upregulation of senescence markers and pro-inflammatory molecules in T cells in response to virus infections have led to the long-standing suggestion that pathogens may initiate or promote neurodegeneration under certain conditions [300, 301]. The relevance of these findings point to a central role for CD8+ T cells which are found

[†] Papadopoulos, D; et al., Accelerated cellular senescence in a model of multiple sclerosis (S50.004); Neurology, April 17, 2017. http://n.neurology.org/content/88/16_Supplement/S50.004

abundantly in MS lesions and where their accumulation has been correlated with the rate of lesion damage [302, 303]. Cellular senescence in T cells has been observed in patients with rheumatoid arthritis and also in MS, implicating perturbation of immune system function associated with senescence in these diseases [304]. Taken together, these findings from mouse models and human patients, suggest cellular senescence in T cells may contribute to neuroinflammation and neurodegeneration in MS.

Microglia and macrophages are known to either hasten or hamper oligodendrocyte differentiation during CNS remyelination [305]. The clearance of debris by these cells from the lesion site is critical in the process of OPC remyelination, but aged microglia demonstrate decreased motility and cellular migration when compared to young [306]. When aged these cells also show decreased phagocytic function, impairing their ability to clear debris, which may induce an arrest in OPC differentiation [307]. Immunosenescence, also termed immune-aging, of both microglia and macrophages could be a potential mechanism for the decreased repair seen in aging [308]. The age-associated decrease in regeneration proficiency can also be attributed to the lessened ability of microglia and macrophages to resolve inflammation. Recently it has been determined that cholesterol, from myelin breakdown, overwhelms aged macrophages and microglia limiting proper clearance functions [309]. The potential for diverse and in some cases opposing role(s) of these cells in natural aging and disease-associated aging has not been fully vetted, but current evidence supports both of these phagocytic cells types as actively involved in degeneration and remyelination and potentially influenced by cellular senescence.

The role of senescence in astrocytes in MS has yet to be studied. While contribution of cellular senescence in other cell types in MS have been suggested, and cellular senescence in astrocytes has been identified in an increasing number of neurodegenerative diseases, including Alzheimer's and Parkinson's disease [229, 249], a similar role for cellular senescence in astrocytes in MS has not yet been reported. Senescent astrocytes are known to express increased glial fibrillary acidic protein (GFAP), associated with increased in pro-inflammatory

cytokines [310]. Increased levels of GFAP in both human and rodent CSF are correlated with age and disease progression in MS, potentially linking senescent astrocytes with progression [311]. Clearance of senescent astrocytes in a mouse model of AD demonstrated a reduction in inflammation, confirming their potential as mediators in MS progression [231]. However, experimental evaluation of the possible roles of senescence in astrocytes will be necessary to draw any conclusions about these reported changes in disease or aging.

OPCs play a crucial role in myelination of the central nervous system. In MS OPCs have been noted to be present within demyelinated lesions yet these progenitor cells remain arrested in an undifferentiated state. In animal studies age has been shown to limit remyelinating potential of OPCs, and in aged rats remyelination can ensue but at a much slower rate when compared to younger rats [312]. Other data have shown that aged OPCs also lack the ability to properly migrate to infiltrate a white matter lesion in which OPCs have been depleted. These data suggest that aging negatively impacts the migratory activities of OPCs, which have been proposed to be due to age-related epigenetic changes [313, 314]. Epigenetics is a dynamic process that has also been found to orchestrate the proper differentiation of OPCs: distinct epigenetic events need to occur for the step-wise progression of OPCs into mature myelinating OLs, and it is now thought that these processes are altered with age and disease [138, 140]. Together, these data suggest that OPCs do not succumb to replicative senescence, but are sensitive to senescence due to chronic stress [315, 316].

Most relevant to this thesis is the emerging importance of cellular senescence in neural progenitor cells. NPCs have garnered attention for their potential to repair myelin and decrease inflammation. NPCs are of particular interest because they have been found within demyelinated lesions, among OPCs, astrocytes and microglia [23, 81, 317]. NPCs are found in greater numbers within the demyelinated lesion areas, when compared to normal appearing white matter (NAWM) or white matter from non-disease individuals. The presence of NPCs spatially related to OPCs in the CNS support the experimental evidence that they contribute to regulation of OPC maturation

and myelin regeneration [118, 318, 319]. NPCs are active participants within the lesion microenvironment and are known to secrete factors that are regenerative, anti-inflammatory, and pro-myelinating [146, 149, 319].

Cellular senescence in NPCs has not been previously explored as a mechanism by which myelination may be limited in MS. Several lines of investigation point to cellular senescence in MS as a plausible disease-related process and also implicate NPCs as a potential culprit cell type. First, the methylation pattern of the $p16^{Ink4a}$ gene has been proposed as an epigenetic risk factor for the development of MS [320]. Second, persistent DNA damage has been reported in cells isolated from human MS lesion tissue [288]. This genotoxic injury could trigger senescence, and the production and secretion of a SASP [321]. Third, inflammation plays a central role in MS and inflammation is known to evoke general epigenetic modifications that can leave cells hypersensitive to DNA damage, and in turn, senescent [321, 322]. Fourth, the chronic demyelination phenotype characteristic of MS and seen prominently in progressive MS patients are correlated with signs of long-standing oxidative stress, inflammation, and even somatic telomere length shortening [288, 323, 324]. Lastly, as mentioned earlier in the chapter, cellular senescence in NPCs provides a mechanism to usurp the expected generation of regenerative factors and replace this with secretion of pro-inflammatory SASP factors. These changes would serve to promote neuroinflammation and keep OPCs in an undifferentiated state (the 'stem cell lock model') [247]. Thus cellular senescence provides a reasonable hypothesis to explain the continuous inflammation in MS disease which ties together epigenetic changes and limited remyelinating potential.

The data in this thesis focuses on my finding that NPCs derived from PPMS patients exhibit cellular senescence not observed in NPCs from aged-matched controls. My data show that this senescent phenotype is responsible for impairing differentiation of OPCs via the SASP. I also report that SASP from PPMS NPCs induces epigenetic changes in naive OPCs, which

account for impaired their proper differentiation. Together, the data in Chapters 4 and 5 of this thesis represent novel findings which demonstrate and implicate cellular senescence in NPCs as a cellular mechanism limiting CNS myelination in this disease.

Chapter 4: iPS-derived Neural Progenitor Cells from PPMS Patients Reveal Defect in Myelin Injury Response

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I. Abstract

Primary progressive multiple sclerosis (PPMS) is a chronic demyelinating disease of the central nervous system (CNS) currently lacking any effective treatment. Promoting endogenous brain repair offers a potential strategy to halt and possibly restore neurologic function in PPMS. To understand how the microenvironment within white matter lesions plays a role in repair we have focused on neural progenitor cells (NPCs) since these are found in lesions in PPMS and have been found to influence oligodendrocyte progenitor cell maturation (OPCs). To better understand the cellular nature of NPCs in PPMS we developed iPS cells from blood samples of PPMS patients and age matched non-disease spouse or blood relative controls. Using these iPS cell lines we determined that the NPCs from PPMS cases provided no neuroprotection against active CNS demyelination compared to NPCs from control iPS lines which were capable of completely preventing injury. Conditioned media (CM) from PPMS NPCs provides no protection to OPCs and prevents maturation of OPCs into oligodendrocytes *in vitro.* We also found that CM from PPMS iPS NPCs elicited patient-specific differences in the response to compounds that should foster oligodendrocyte (OL) maturation. Together, these data establish a new model for understanding the nature of myelination defects in PPMS which may lead to novel targeted approaches for preventing demyelination in these patients.

Keywords

induced pluripotent stem cells, neural progenitor cells, primary progressive multiple sclerosis, oligodendrocytes

Abbreviations

central nervous system, CNS; conditioned media, CM; cuprizone, cup; embryonic stem cells, ESCs; induced pluripotent stem cells, iPSCs; multiple sclerosis, MS; neural progenitor cells, NPCs; oligodendrocytes, OLs; oligodendrocyte progenitor cells, OPCs; primary progressive multiple sclerosis, PPMS; wild type, WT

II. Introduction

Primary progressive multiple sclerosis (PPMS) is the most severe form of MS as it is distinguished from other forms of this disease by the rapid development of disability often without relapses [16, 325]. Disability in all forms of MS is associated with the loss of CNS myelin and the ensuing disruption of axon conduction, decline of neuronal functions, and eventual neurodegeneration [326]. Promoting remyelination is viewed as a promising approach to restore salient brain functions in MS patients [327]. This approach holds particular interest for treating PPMS as current immunomodulatory therapies offer little benefit to these patients [328, 329]. The efficacy of any prospective remyelinating therapy will rely on the potential of resident OPCs in their propensity to differentiate into mature myelinating OLs within the lesioned environment. For reasons that remain unclear, OL differentiation in PPMS is interrupted, inadequate, or simply fails, resulting in chronically demyelinated lesions. Discerning whether neural progenitor cells from PPMS patients can appropriately respond to myelin injury and prevent demyelination is critical to the translation of experimental therapies to the clinic.

Studies of myelin lesion pathology have identified premyelinating OPCs within the lesion, suggesting an innate potential for remyelination that is somehow limited by the disease or lesion environment. Within a demyelinated lesion there are many cell types, including astrocytes, microglia, and neural progenitor cells (NPCs) [23, 81, 317, 330, 331]. NPCs are of particular interest in term of understanding innate limits on remyelination because they too are activated and recruited into demyelinated lesions in MS, and NPCs as are known to regulate OPC maturation and myelin regeneration [318, 319]. This idea is supported by experimental evidence that murine NPCs when transplanted into mouse models of demyelination are potently anti-inflammatory and neuroprotective [146-148], and can also promote OL differentiation of endogenous OPCs [319, 332]. These data suggest NPCs are active participants to CNS myelination and contribute to the environment of a myelin lesion and its potential for CNS

remyelination [319], and yet, in PPMS, remyelination fails. We hypothesized that NPCs are a cause of remyelination failure in PPMS.

Induced pluripotent stem (iPS) cells represent a unique opportunity to study the mechanism of pathology involved in PPMS, and to specifically address the functional contribution of neural progenitor cells towards demyelination in PPMS. No prior study has previously examined NPCs from MS patients, however, patient-derived iPS cells have been recently shown to exhibit efficient differentiation into oligodendrocytes *in vitro* and *in vivo* using the shiverer mouse model, which lacks myelination in the central nervous system [157, 333]. These results indicated that iPS cells from PPMS cases have the potential to respond appropriately to the necessary cues required for OL differentiation, suggesting that limits on OPC maturation inherent to PPMS may reside in the function of other cell types in the lesion. We have previously reported that NPCs derived from human embryonic stem (ES) cells were capable of attenuating active demyelination during cuprizone-induced demyelination in mice [156]. In the present study we developed NPCs from PPMS and control patient blood samples and tested them in the context of an active demyelinating lesion. These iPS lines were tested to determine whether NPCs from PPMS patients were equally capable of promoting myelination and neuroprotection *in vivo.* Our results demonstrate a dramatically reduced capacity of PPMS NPCs for regeneration in demyelinating lesions, which may inform future reparative strategies for treating this disease.

III. Materials and Methods

iPS Cell and NPC Culture Derivation. Procedures involving patients and donors were approved by the Institutional Review Boards at both study sites prior to collection of any blood samples (Protocol IE-13-069-2). To generate iPS cells, blood samples were collected from patients with confirmed diagnosis of PPMS and spouses or blood relatives without any identified neurological or autoimmune diseases (control), at either the University of Connecticut Health Center (Farmington, CT) or The Mandell MS Center at St. Francis Hospital (Hartford, CT) (Table 4.1). Using the Sendai virus, multiple clones of each line were generated by the UConn Health Center Stem Cell Core (Supp. Table 1). The iPS cells were then plated onto mouse embryonic fibroblasts (MEFs) and were fed iPS cell medium consisting of DMEM/F-12 (Life Technologies), supplemented with 20% KnockOut Serum Replacement (KSR) (Life Technologies), 0.5% L-glutamine (Life Technologies), 1% penicillin/streptomycin (Life Technologies), 8 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems), and 0.1 μM/mL 2-mercaptoethanol (Sigma-Aldrich). The pluripotency and genomic integrity of all iPS cell lines (Table 4.1) was validated by immunocytochemical analyses for Oct4 and SSEA4 and karyotyping analyses (Supp. Fig. 4.4A). Results indicated successful and uniform reprogramming across all lines with colonies from each line exhibiting similar morphology (Supp. Fig. 4.4A). To differentiate the iPS cells to NPCs, neural stem cell medium (N2/B27), that consisted of neurobasal medium (Life Technologies), 2% B27 (Life Technologies), 1% N2 (Life Technologies), 1% L-glutamine, 1% insulin-transferrin-selenium (Life Technologies) and 1% penicillin/streptomycin with noggin (Fisher Scientific) (250 ng/mL) was applied to the multiple clones of each iPSC line for 10 days. Noggin is then removed, cells are plated onto laminin (5 μg/mL, Sigma Aldrich), and fed NPC medium for another 6 to 11 days. When NPCs were ready for injection into the mice they were collected and counted in the NPC medium right before injection. Immunocytochemistry for the iPS cells was performed by the University of Connecticut Health Center Stem Cell Core, including the phase contrast images,

and staining for Oct4 and SSEA4. Karyotyping was performed by the UConn Induced Pluripotent Stem Cell and Chromosome Core (Supp. Fig. 4.4A).

Cuprizone-induced Demyelination and NPC Administration. All procedures involving the use of mice were approved by the Institutional Animal Care and Use Committee at the University of Connecticut Health Center, and in accordance with guidelines set forth by the National Research Council of the National Academies Guide for the Care and Use of Laboratory Animals. Male C57BL/6 mice, between the ages of six to eight weeks, were fed cuprizone (0.2% w/w, Sigma Aldrich) in powdered rodent lab chow for four consecutive weeks. Two weeks after the initiation of the cuprizone diet, the mice were injected intravenously into the tail vein with either NPC media (100 µL), or an equivalent volume containing 5 x 10^5 control NPCs or PPMS NPCs (n=3-5 mice group/line). Multiple clones of either line 1.1 or 2.1 were injected into mice as the control lines, and multiple clones of either line 1 or 2 were injected into mice as the PPMS lines. For NPC injection clones were grown together with their respective line and injected together. Mice were maintained on the cuprizone diet for an additional two weeks after cells were injected and then mice were euthanized for brain tissue collection. Brains from saline-perfused animals were fixed in 10% formalin for paraffin embedding and histological analyses. Electron microscopy was performed on tissues from mice also perfused with 4% paraformaldehyde (Sigma Aldrich) and 2% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M cacodylate buffer, and their brains post-fixed in the perfusion solution.

Immunocytochemistry (ICC). NPCs were plated on laminin-coated coverslips (5 µg/mL, Sigma Aldrich), and fixed using 4% paraformaldehyde. Following fixation they were permeabilized using triton-X100 (0.5%, Sigma Aldrich). They were then stained using the following primary antibodies: GFAP (1:1000; Dako, Carpinteria, CA, USA), Musashi 1 (1:250; Abcam, Cambridge, MA, USA), MAP2 (1:1000; Abcam, Cambridge, MA, USA), Tuj1 (1:1000; Abcam, Cambridge, MA, USA),

smooth muscle actin (1:100 Abcam, Cambridge, MA, USA), and Oct4 (1:100; Abcam, Cambridge, MA, USA). All NPC lines and clones were validated with this staining protocol (Supp. Fig. 4.1 and Table 4.1).

Apoptosis Detection (TUNEL). Detection of apoptosis was carried out using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland) according to the manufacturer's protocol. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Sections were digested with 15 µg/mL proteinase K (BD Biosciences, San Jose, CA, USA) for 30 minutes, washed in PBS, and incubated with the label and enzyme solution provided in the kit for one hour at 37°C. Sections were washed and then mounted using Cytoseal (Fisher Scientific, Pittsburgh, PA, USA). Negative and positive controls were prepared as described. Coronal brain tissue sections (between 1.10 mm rostral to bregma and 1.7 mm caudal to bregma) were used for all analyses. Sections were examined at 60x magnification and images of the left, right, and medial corpus callosum were collected using identical exposure settings using computer-assisted image analysis software (Olympus IX71, Northern Eclipse, EMPIX Imaging). The number of TUNEL+ cells was determined in each image (n = 2-4 mice/treatment group, n = 3-5 sections/mouse).

Electron Microscopy (EM). EM was performed on microdissected tissues from the midline aspect of the corpus callosum and postfixed in 1% OsO_4, as previously described [334]. Samples were dehydrated using graded ethanols, stained with uranyl acetate, and embedded in Poly/Bed812 resin. Ultrathin (0.1 µm) sections from each tissue block were cut and visualized using an electron microscope (80 kV, 5000x magnification). G-ratio analyses were performed using ImageJ software (National Institute of Health) [335] to determine axon to myelinated axon diameters for each condition.

Isolation of Rat OPCs and Treatment with NPC Conditioned Media. OPCs were obtained from the cerebral cortices of neonatal rat pups (postnatal day 0-2), and were plated on poly-l-ornithine coated coverslips, as previously described [336]. OPCs were then cultured in control or PPMS NPC conditioned media (CM) for two days. The conditioned media experiments were performed in quadruplicate technical replicates and repeated in triplicate using separately developed cultures (i.e. unique biological replicates). Conditioned media was taken from the NPCs after 48 hours on 80% confluent cells, at least 4 days after passage, and immediately spun down to remove debris then frozen at -80°C. OPCs were treated with NPC CM for 48 hours and then were fixed and stained for Olig2 (1:500; Millipore, Temecula, CA, USA) and MBP (1:500; Millipore, Temecula, CA, USA). In order to test OPC protection by NPC CM during an *in vitro* stressor OPCs were treated with 500 μM of glutamate (Sigma) for 12 hours in NPC CM from control or PPMS patients. After 12 hours the cells were washed with PBS and the Live/Dead Viability/Cytotoxicity Kit (Invitrogen) was performed according to the manufacturer's instructions. OPC proliferation was measured using BrdU (10 μM, Millipore) applied for four hours at the time of applying NPC CM [337]. After treatment, cells were washed, fixed, and stained for Olig2 and BrdU (1:250; eBioscience, San Diego, CA, USA). For pharmacological treatments, cultures were grown in NPC CM for 2 consecutive days with addition of either benztropine (Sigma, 1 μM), clemastine (Sigma, 1 μM), clobetasol (Millipore, 5 μM), or miconazole (Sigma, 1 μM). For all experiments five fields of view at 20x using identical image capture settings were assessed by an experimenter blinded to treatments (Olympus IX71, Northern Eclipse, EMPIX Imaging). For analysis of OPC differentiation, all Olig2+ cells and MBP+ cells were counted and the percentage of MBP+ cells calculated, for cell death analysis a percentage of dead cells was calculated from the total cell number, and proliferation was evaluated by analysis of BrdU positive cells as calculated as the percent labeled of total Olig2+ cells (n = 3 biological replicates for each cell line).

Statistical Analysis. Data was analyzed by Student's t-test or one-way ANOVA, where appropriate and as indicated, using GraphPad Prism version 6 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com). Differences were considered significant when $P < 0.05$. Data are presented as mean ± SEM.

IV. Results

1. PPMS NPCs Fail to Provide Neuroprotection Against Cuprizone-Induced Demyelination.

We hypothesized that development of PPMS-patient iPS lines would enable us to evaluate and compare the ability of disease-specific cells to foster myelination. To test this hypothesis, we examined whether differentiated NPCs from control and PPMS patients could foster CNS myelination *in vivo*. We had previously demonstrated that human embryonic stem cell-line derived NPCs potently attenuated demyelination in mice treated with cuprizone [156]. This cuprizone model offered several advantages to study the cellular effects of the iPSC-derived NPCs on CNS myelination. First, this model induces robust, reproducible, and predictable CNS demyelination mediated by OL cell death [156, 338, 339]. Cuprizone-induced demyelination is most notable in heavily myelinated areas, including the corpus callosum providing a clearly defined *a priori* locus for analysis [104, 156]. Second, cuprizone-induced demyelination is not dependent on the adaptive immune response, which in turn tests the propensity of these cells to foster myelination exclusive of their potential to dampen autoreactive T cells which is inherent to other experimental models of demyelination [146]. Most importantly, our previous study had validated this model for its utility as an acute *in vivo* assay to test the myelinating potential of human cells [156].

Wild type (WT) C57BL/6 mice were fed cuprizone for two consecutive weeks and then randomly assigned to receive either NPCs from PPMS patients or control lines (Fig. 4.1A, Table 1). Control and PPMS NPCs injected into cuprizone-fed mice consisted of three clones per line with multiple animals per treatment group (n=3-5 per group/line; Supp. Table 1). Mice were maintained on cuprizone for an additional two weeks at which point brain tissues were collected for quantitative assessment of myelinated axons in the corpus callosum (Fig. 4.1A). Electron microscopy of myelinated axons in the corpus callosum revealed a profound loss of myelination in cuprizone-fed mice (Fig. 4.1B), resulting in a significant increase in the axon g-ratio (0.70 naïve mice vs. 0.78 cuprizone mice; Fig. 4.1C and D upper panels). Analysis of the corpus callosum in cuprizone-fed mice administered control NPCs revealed a marked improvement in compact

myelination, while PPMS NPC-treated mice exhibited few compactly myelinated axons, that was not different from lesion-control animals (Fig. 4.1B), and exhibited a significant elevation in myelinated axon g-ratios (Fig. 4.1C and D lower panels). Analyses on the proportion of axons that were unmyelinated in the corpus callosum confirmed a demyelinating effect of cuprizone treatment (Fig. 4.1E upper panel). Administration with control NPCs resulted in a marked preservation in the number of myelinated axons, while PPMS NPC treatment resulted in significantly fewer myelinated axons (Fig. 4.1E lower panel). Cell death analyses within the corpus callosum revealed a significant increase in TUNEL+ cells in cuprizone-treated animals, while cuprizone-fed animals that were also administered control NPCs did not differ from the naive mice (Fig. 4.1F). NPCs from PPMS cases failed to provide neuroprotection from cuprizone treatment (Fig. 4.1F). The same amount of human nuclear antigen positive NPCs enter the corpus callosum from either the control or PPMS NPC lines (Supp. Fig. 4.5). These results suggest that unlike NPCs from control cases which increase the number of myelinated axons in the corpus callosum, NPCs from PPMS patients failed to provide any benefit in preserving compact CNS myelination during active demyelination produced by cuprizone treatment.

We had previously reported that hES-NPCs given to cuprizone-treated mice exhibited multiple cell fates in the corpus callosum, including differentiation into GFAP+ astrocytes and O1+ mature oligodendroglia [156]. We also examined the cell fates of the NPCs in tissues of control NPC and PPMS NPC-treated mice. We determined that the fate of NPCs from control lines tended to differentiate towards an oligodendrocyte phenotype, whereas PPMS NPCs exhibited significantly fewer oligodendrocytes but significantly more astrocytes (Supp. Fig. 4.5). The reason for these differences in NPC fate are not understood but likely relate to the differences in CNS myelination observed between control and PPMS NPC-treated groups.

2. PPMS NPCs Fail to Protect Rat OPCs from Toxicity and do not Support Oligodendrocyte Differentiation *in vitro*.

The failure of PPMS-NPCs to prevent demyelination of the corpus callosum during cuprizone-treatment suggested a failure of the NPCs to confer neuroprotection. To test this further, we cultured primary rat OPCs, treated these with conditioned media (CM) from either control or PPMS NPCs and the applied glutamate as an excitotoxic stimulus to induce cell death (Fig 4.2A). Cell death, was measured and compared between treatment groups (Fig 4.2B). Application of glutamate (500 μM/12 hrs) [340] was found to induce comparable amounts of cell death in both control media (N2/B27 media) or CM from control NPC cultures. In contrast, OPCs grown in CM from PPMS NPCs exhibited twice as much cell death in response to glutamate as either control condition (Fig 4.2B). These results suggested that PPMS CM exerted a negative influence on OPCs which may not be limited to failure to protect against a noxious stimulus. We hypothesized that PPMS NPC CM may also negatively impact OPC differentiation. To test the possibility that PPMS NPCs differed in their ability to support OPC differentiation we collected conditioned media (CM) from PPMS NPC and control NPC cultures. These media were used to differentiate primary A2B5+ oligodendrocyte progenitor cells *in vitro* for two days (Fig. 4.2C). Conditioned media collected from control NPCs was found to promote differentiation of A2B5+ OPCs into mature MBP+ oligodendrocytes (OLs) (Fig. 4.2D and E), while CM from PPMS NPCs failed to promote successful OL differentiation, based on the percentage of MBP positive cells in the culture. Out of all three PPMS lines, the NPC CM significantly inhibited differentiation when compared to the NPC CM of the spouse or sibling control (Fig. 4.2E). The number of Olig2+ cells in each culture condition did not differ (Fig 4.2E), nor did the proliferation of OPCs differ between control or PPMS CM treated cultures (Fig. 4.2E). Together, these data indicated that CM from PPMS NPCs did not kill OPCs nor did it induce proliferation of OPCs which could have accounted for the reduced differentiation in these culture conditions. Thus, PPMS CM was less able to promote oligodendrocyte differentiation than control NPCs.

3. NPC CM Mediated Oligodendrocyte Differentiation Exhibited Patient-Specific Responses Towards Potential Remyelination Compounds.

We next examined the impact of PPMS NPCs on the efficacy of recently identified compounds reported to stimulate remyelination [110, 163, 164]. First we show that in unconditioned N2/B27 media there is an increase in MBP positive cells with drug treatment as previously reported (Fig. 4.3A). Next, we tested whether conditioned media from our human control NPC and PPMS NPC cultures would impact the efficacy of either benztropine[164], clemastine [110], miconazole, or clobetasol [163] to stimulate OPC differentiation. To test this we employed the same cell culture paradigm as above (Fig. 4.3B), and applied all compounds as previously reported. We found that when applied to OPCs grown in CM from control lines compounds elicited increased maturation of A2B5+ OPCs to MBP positive cells (Fig. 4.3C and D). In contrast, administration of these compounds to OPCs cultured in CM from PPMS NPCs exhibited selective effects on OL maturation that varied by patient (Fig. 4.3C and E). In three examples, PPMS line 1 only displayed a significant improvement in OPC maturation in response to clemastine and miconazole, whereas OPCs grown in CM from PPMS line 2 exhibited modest improvement in response to clobetasol but not the other compounds tested, and lastly OPCs grown in CM from PPMS line 3 exhibited maturation in response to clemastine, clobetasol, and miconazole, but not benztropine (Fig. 4.3C and E). These data indicate that CM from PPMS NPCs influence the responsiveness of OPCs to pharmacological stimulation of differentiation observed in cells exposed to control NPC CM.

V. Discussion and Conclusions

A therapeutic goal to treat MS is the preservation of CNS myelin and to restore salient functions to demyelinated axons in MS patients. Accumulating evidence supports a prominent role for NPCs as important regulators of neuroprotection and remyelination in the MS brain. Our findings are the first to report that NPCs developed from PPMS patients exhibit inherent defects when compared to control NPCs. PPMS NPCs failed to provide neuroprotection during an active myelin injury *in vivo*, and secondly, they were found not to support OPC differentiation *in vitro*. These findings indicate that NPCs from PPMS patients may be inherently different in terms of their myelinating support compared to NPCs of control cases. Our findings also demonstrated that PPMS NPCs from different patients were inherently distinct from each other in that their response to potential pro-myelinating drugs varied from patient to patient, and thus suggests a uniqueness to the regenerative potential of each PPMS patient. A future potential development of these patient-specific iPS cell lines could be individualized assessment of NPC functions for personalized treatment to assess neuroprotection and promote remyelination. Thus, our application of iPS NPCs, and CM, may represent a novel approach of discovering patient-specific remyelinating therapies when used in conjunction with wider drug screening assays.

Studies of myelin pathology in MS cases have identified premyelinating OPCs within demyelinated lesions [341]. This observation indicates an endogenous potential for NPCs as a source of limiting factors related to disease and/or the lesion microenvironment [342]. That NPCs are activated and found within demyelinated lesions in MS has also prompted attention on NPCs as possible regulators of myelin regeneration [318, 319]. Experimental evidence using wild type (non-disease) murine NPCs has found that when transplanted into mouse models of demyelination can have potent anti-inflammatory qualities and provide neuroprotection [146, 147]. These wild type murine NPCs can also promote OL differentiation from endogenous OPCs [319, 332]. The benefit of NPCs in these experimental models of CNS demyelination is (in part) mediated by secreted factors [150-153], such as growth factors that can foster myelin repair [154,

155]. Consistent with this we have previously demonstrated that human ESC-derived NPCs attenuated demyelination in the cuprizone model following intravenous administration [156]. The effect of the human NPCs in this model were found to be neuroprotective and not limited to cell replacement, consistent with the notion that a function of NPCs is to maintain or restore homeostatic processes [343]. Our *in vivo* fate analysis of injected NPCs in the cuprizone mice identified significant differences between control and PPMS lines. We noted that while control NPCs differentiated into some astrocytes and mostly oligodendrocytes, PPMS NPCs preferentially differentiated into astrocytes only (Supp. Fig. 4.5). While we do not fully understand the physiological reasons for these differences, we hypothesize that these differences relate to the reduced ability of PPMS NPCs to promote CNS myelination. Astrocytes are known to influence the fate of OPCs and myelination and reactive astrocytes are a pathological feature of CNS myelin lesions. This may suggest that the bias toward an astrocytic lineage in the PPMS lines could reflect a central role for astrocytes in remyelination in PPMS. Alternately, previous findings have also shown that genotoxic injury to NPCs can induce a bias toward astrocyte differentiation [344]. Within MS lesions DNA damage has been observed [288]. These data may suggest that disease-related changes in PPMS could have resulted in a bias of the NPCs from these patients to then differentiate towards an astrocytic fate. Future studies should will be needed to better understand this difference in cell fate as it relates to the function of NPCs and their neuroprotective potential in PPMS. Our results add to this body of work and suggest that NPCs in PPMS are fundamentally different and have an effect on mediating changes on the local microenvironment through secreted factors that negatively impact OPC maturation. If one were to translate these *ex vivo* findings to an MS lesion microenvironment, our data would suggest that NPCs in PPMS are a contributing factor to demyelination that may deplete OPCs and thereby limit endogenous remyelinating potential.

Our findings using patient specific iPS cells indicate that the function of NPCs in PPMS is modified by disease and may be a critical determinant of myelin repair. For instance, we show

that PPMS-CM exhibited very little differentiation on OPCs *in vitro*. This may have been due to reduced growth factor production by the PPMS NPCs, which may have then contributed to the stunted maturation of MBP+ OLs. One approach we tested was the potential for iPS cells to be used to identify personalized remyelinating compounds to treat PPMS. Our data determined that four FDA approved drugs recently found to aid OPC maturation had significant differences in efficacy when OPCs were grown in conditioned media from PPMS NPCs. Clemastine, one of the drugs that was found to enhance oligodendrocyte differentiation, is an antihistamine and anticholinergic compound that acts as an antagonist for muscarinic receptors [110, 164], while another drug, miconazole, an antifungal agent, acts as an antagonist towards glucocorticoid receptors [345]. Based on our results, clemastine may offer therapeutic potential for one patient line tested, and yet none of the compounds tested effectively enhanced OPC differentiation for the other. These results suggest potentially important differences between patients and in how NPCs from these different individuals with PPMS have impaired OPC maturation. Moreover, using the NPCs from PPMS iPS cells we also may exploit these individualized differences to better identify and understand what process(es) limit remyelination, that may also serve to distinguish PPMS from other forms of MS. This NPC-based approach may be adopted to optimize the potential of identifying specific compounds for different patients, or groups of patients, that effectively stimulate OPC differentiation. Using a personalized iPS-cell based screen would be an innovative approach that offers the potential for promoting neuroprotection, fostering remyelination and restoring salient functions for PPMS patients.

The results of this study complement previous findings by Douvaras and colleagues who reported that fibroblast-derived iPS cells from PPMS patients were capable of differentiating into myelinating oligodendrocytes *in vitro* and *in vivo*, using the shiverer mouse model [157]. Previous work had determined that PPMS iPS cells were capable of OL differentiation and maturation [157], however our findings reveal restricted and limited promyelinating potential of PPMS NPCs in the corpus callosum during active demyelination. It is important to point out that there are several

important differences that distinguish this study and the previous report. First, we focused on the NPC as the cell type of interest and found that PPMS NPCs exhibited a dramatic effect on oligodendrocyte differentiation. Secondly, we challenged PPMS NPCs in the context of an active demyelinating lesion and found a significant defect in the neuroprotective potential of PPMS-derived cells. Third, we reprogrammed cells from blood whereas the previous study used skin fibroblasts. Taken together, results from iPS cell studies of PPMS would indicate that cells form these patients may have the potential to form myelin but that the potential is limited by the complex cellular interplay in the diseased CNS.

The cause and biological mechanism underlying the pathology of PPMS is presently unknown. Our development and testing of PPMS NPCs provide new information and an important opportunity to better understanding PPMS, but perhaps other progressive forms of MS as well. Future studies examining the physiological effect of iPS-derived NPCs from secondary progressive [346] and also relapsing-remitting forms of MS would be warranted to determine whether NPC function is compromised across only progressive forms, or all clinical forms of MS. Our data suggest NPCs from PPMS may contribute to an inherent defect in CNS myelination in PPMS. It remains unclear whether these important differences in myelinating potential are innate to individuals who then develop PPMS, or are a result of maladaptively acquired changes resulting from the experience of having PPMS. These findings provide a new perspective from which we may better understand this disease and develop new approaches for abating or reversing the insidious course of PPMS.

VI. Acknowledgements

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VII. Figures

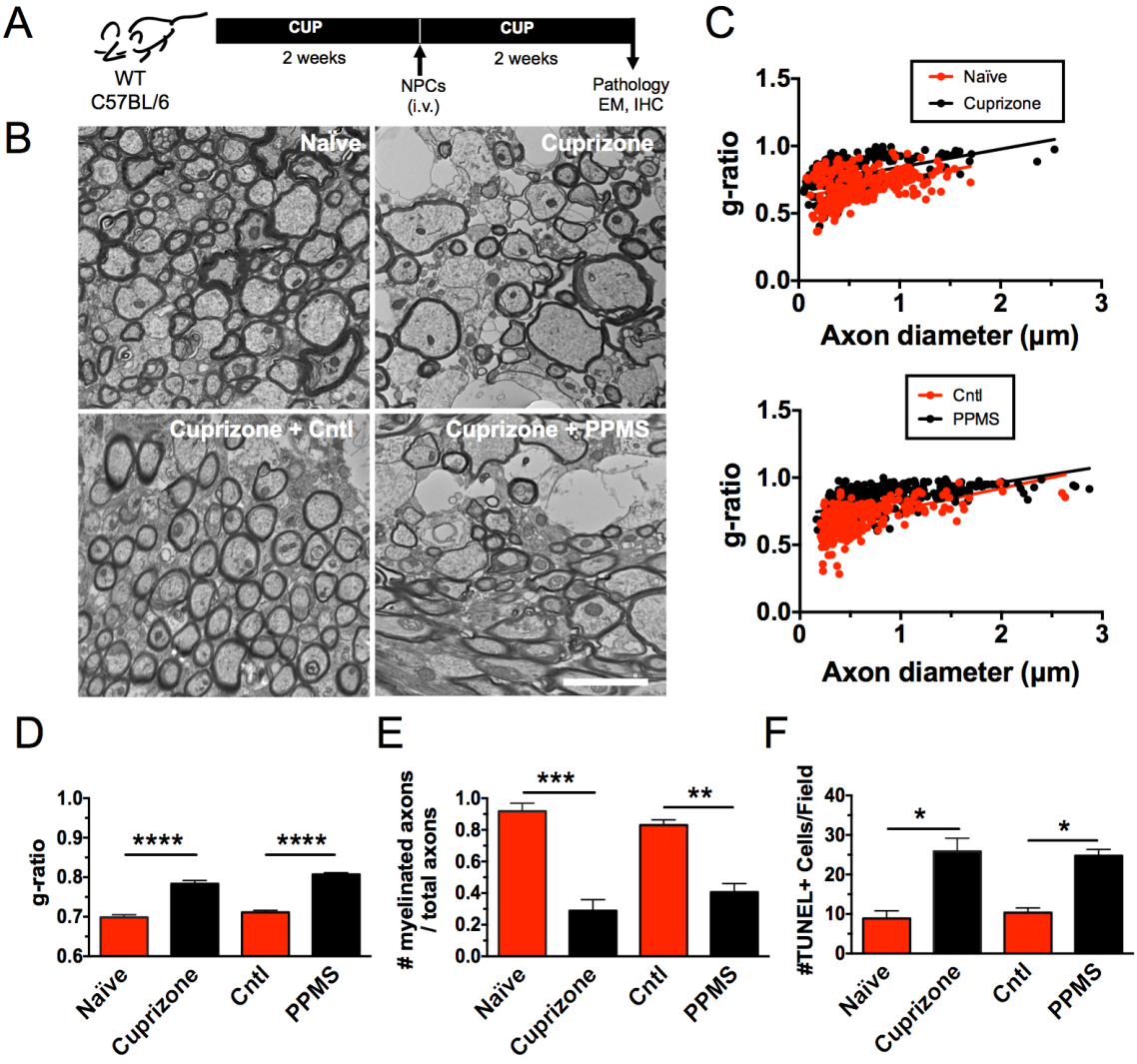
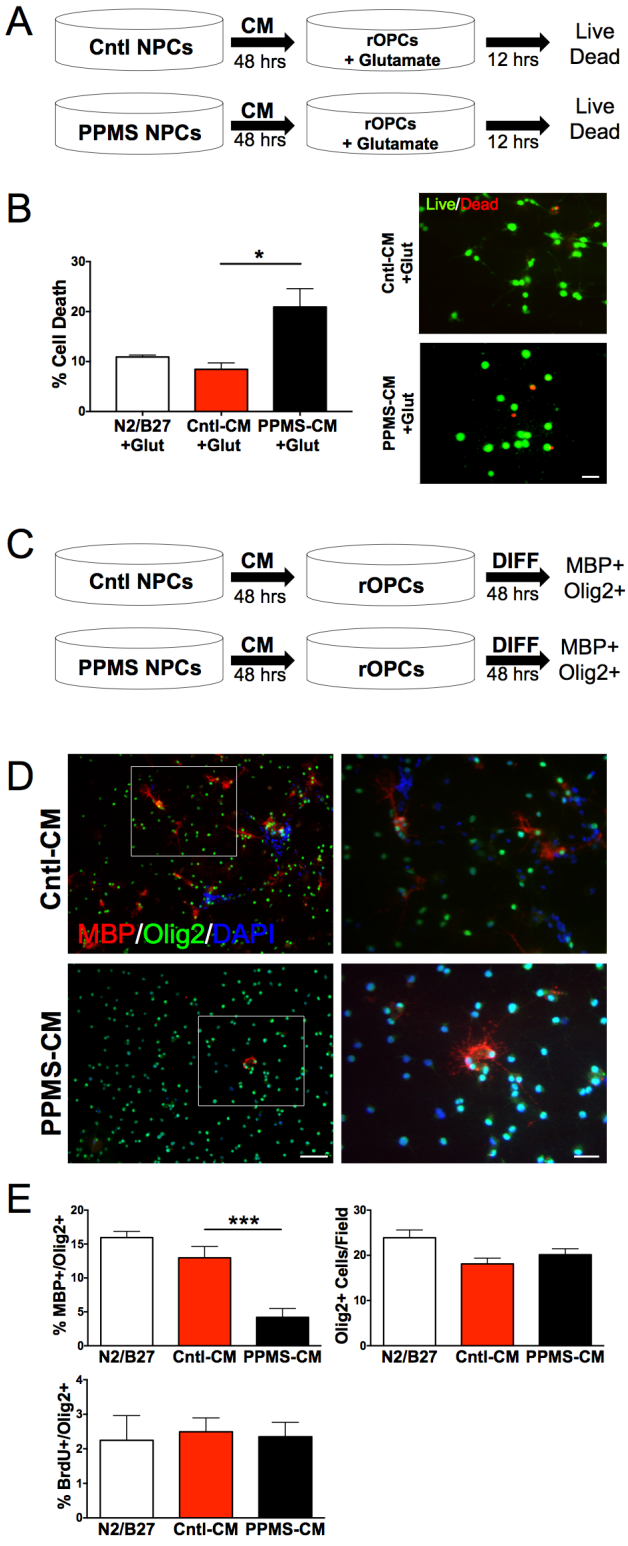


Figure 4.1

Figure 4.1. Reduced compact myelination in the corpus callosum of cuprizone-treated mice administered PPMS NPCs. *(A)* Experimental timeline for the treatment of cuprizone-fed mice with iPS-derived NPCs. Wild type (WT) C57BL/6 mice were fed cuprizone ("cup") for 2 weeks, injected with iPS derived NPCs (i.v.), and after an additional 2 weeks of cuprizone administration brain tissues isolated for pathology**.** *(B)* Representative electron microscopy (EM) images (5000x magnification) of compact myelination in the corpus callosum of naïve mice, cuprizone-fed mice, and cuprizone-fed mice injected with either control NPCs, or cuprizone-fed mice injected with PPMS NPCs. Scale bar, 3 μm. *(C)* Scatter plots of myelinated axon g-ratios in each treatment group comparing naïve vs cuprizone with NPC treatment and both NPC treated groups (control NPCs vs PPMS NPCs). *(D)* Average myelinated axon g-ratios (mean ± SEM) for each treatment group (**** $P < 0.0001$, one-way ANOVA). *(E)* Quantification of myelinated and unmyelinated axons in the corpus callosum of each treatment group, as indicated (** $P < 0.01$, *** $P < 0.001$, one-way ANOVA). Data points represent individual g-ratios for axons where naïve n = 262 axons, cuprizone n = 221 axons, control NPC n = 256 axons, and PPMS NPC n = 303 axons. *(F)* Quantification of TUNEL+ cells in the corpus callosum of each treatment group, as indicated (* $P < 0.05$, one-way ANOVA). Naive = mouse fed no cuprizone; Cuprizone = mouse fed cuprizone; Cntl = mouse fed cuprizone and injected with control NPCs (either line 1.1, consisting of clones 1, 2, and 5 together; or line 2.1, consisting of clones 2, 3, and 6 together]); PPMS = mouse fed cuprizone and injected with PPMS NPCs (either line 1, consisting of clones 1, 4, and 3 together; or line 2 consisting of clones 1, 2, and 4 together).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Cntl NPCs
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+ Glutamate
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PPMS NPCs
CM
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Cntl-CM
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PPMS-CM
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0
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N2/B27
Cntl-CM
PPMS-CM
Olig2+ Cells/Field
30
20
10
0
N2/B27
Cntl-CM
PPMS-CM
% BrdU+/Olig2+
4
3
2
1
0
N2/B27
Cntl-CM
PPMS-CM

Figure 4.2

Figure 4.2. PPMS NPCs do not protect OPCs and do not support oligodendrocyte differentiation. *(A)* Control and PPMS NPCs were grown in culture for at least 48 hours at 80% confluency and conditioned media (CM) collected. Primary rat OPCs (rOPCs) were grown in CM from control NPCs or PPMS NPCs with 500 μM glutamate treatment for 12 hours and cell death was assessed. *(B)* The percent cell death of OPCs treated with either control CM or PPMS CM in the presence of glutamate. PPMS CM did not protect OPCs from glutamate toxicity. (* $P < 0.05$, one-way ANOVA). Representative pictures of live cell/dead cell assay. Scale bar, 50 μm. *(C)* Control and PPMS NPCs were grown and CM collected as in A. Primary rat OPCs (rOPCs) were grown in CM from control NPCs or PPMS NPCs for 48 hours and OL maturation was assessed. *(D)* Representative images of mature oligodendrocytes (MBP+/Olig2+) resulting from either control CM or PPMS CM. Scale bar, 200 μm, magnified panel scale bar, 50 μm. *(E)* Quantitative comparison of OL maturation of OPCs grown in either non-conditioned N2/B27 media, PPMS CM, or control CM determined that the proliferation of Olig2+ cells was identical between each treatment condition, while OL maturation (Olig2+/MBP+) was significantly lower in OPCs grown in PPMS CM. No differences in OPC proliferation (BrdU) was observed in control (N2/B27), or CM from either control NPCs or PPMS NPCs. n = 12 independent cultures per treatment (*** $P < 0.001$, one-way ANOVA). N2/B27 = non-conditioned NPC media; Cntl-CM = CM from control lines; PPMS-CM = CM from PPMS lines.

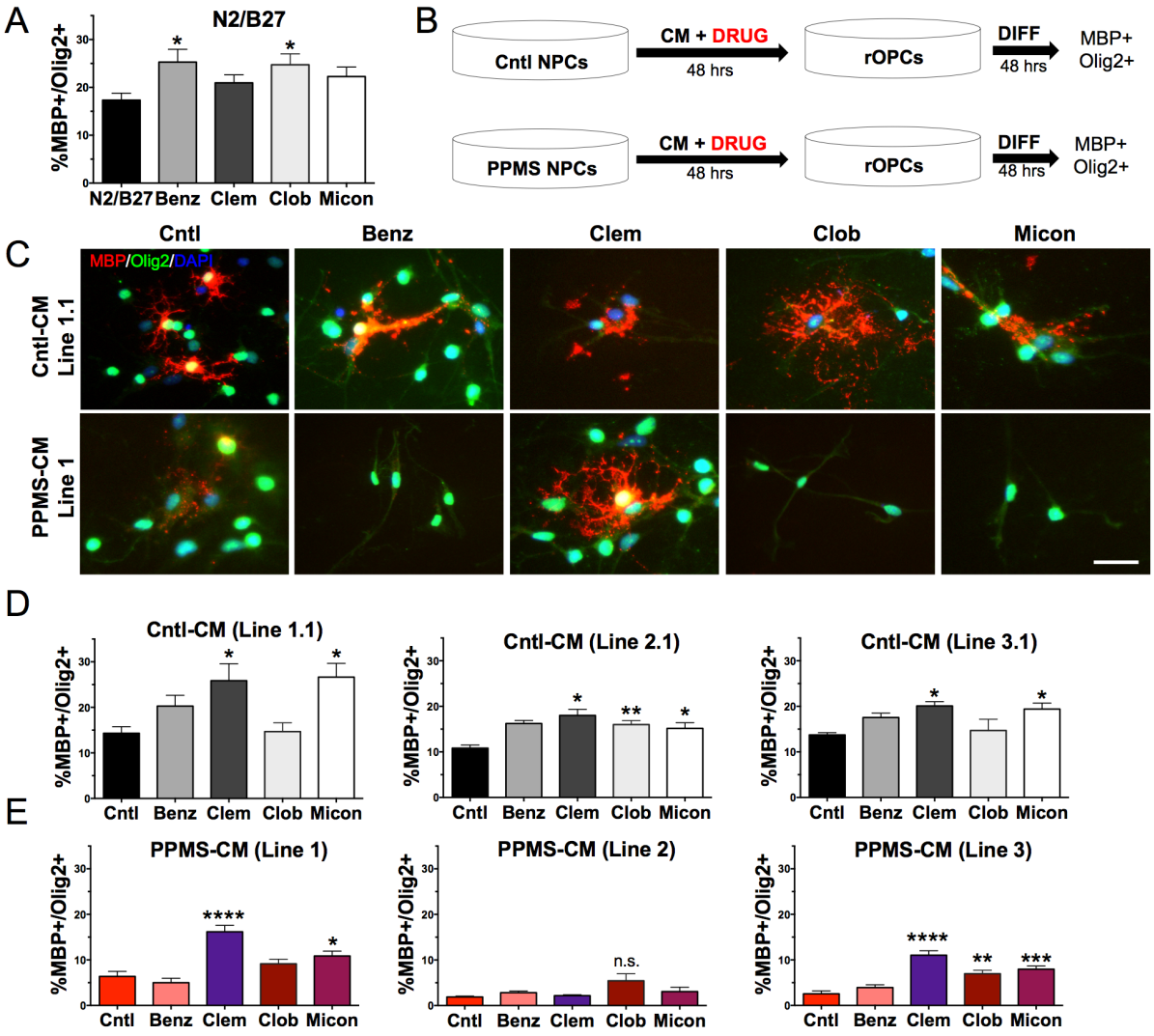

A
N2/B27
%MBP+/Olig2+
N2/B27 Benz Clem Clob Micon
B
Cntl NPCs
CM + DRUG
48 hrs
rOPCs
DIFF
48 hrs
MBP+
Olig2+
PPMS NPCs
CM + DRUG
48 hrs
rOPCs
DIFF
48 hrs
MBP+
Olig2+
C
Cntl
Benz
Clem
Clob
Micon
MBP/Olig2/DAPI
Cntl-CM Line 1.1
PPMS-CM Line 1
D
Cntl-CM (Line 1.1)
Cntl-CM (Line 2.1)
Cntl-CM (Line 3.1)
%MBP+/Olig2+
Cntl Benz Clem Clob Micon
E
PPMS-CM (Line 1)
PPMS-CM (Line 2)
PPMS-CM (Line 3)
n.s.

Figure 4.3

Figure 4.3. Individualized patient-specific differences to promyelinating drugs. *(A)* Quantification of OL maturation in OPCs grown in non-conditioned N2/B27 media with application of promyelinating drugs: benztropine ("Benz"), clemastine ("Clem"), clobetasol ("Clob") or miconazole ("Micon"), as indicated. *(B)* Control and NPC CM was collected after 48 hours at 80% confluency. rOPCs were grown in CM from either control NPCs or PPMS NPCs in addition to either of the four promyelinating drugs, and after 48 hours OL maturation was assessed. *(C)* Representative images of Olig2+ OPC-lineage cells (green) and MBP+ OLs (red) after 48 hours of treatment with either control NPC CM or PPMS NPC CM alone or in combination with application of four promyelinating drugs. Scale bar, 200 μm. *(D)* Quantification of OL maturation (%MBP+/Olig2+) in OPCs grown in control NPC CM and the measurable effect on OL maturation of the four promyelinating compounds. *(E)* Quantification of OL maturation in OPCs grown in PPMS NPC CM and the measurable effect on OL maturation of the four promyelinating compounds. n = 3 independent cultures per treatment from 8 technical replicates/treatment. Values are means ± SEM. (* $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$ **** $P < 0.0001$, one-way ANOVA).

Table 4.1. iPS Cell Line Derivation from PPMS Patients and Spousal or Blood-Relative Controls. Information on PPMS patients (n = 3), and either their spouses or sibling (n = 3), that provided blood samples used to develop new iPS cell lines. Average age of each group did not differ (*P* = 0.876; PPMS mean = 56; control mean = 57). Average duration of PPMS among the patient samples at the time of collection was 7.6 years with a mean 5.3 EDSS score. EDSS, expanded disability status scale.

Line	Gender	Age	Disease	Diagnosis	Relationship	EDSS Score
1	M	61	PPMS	5 Years		5
2	F	62	PPMS	14 Years		7.5
3	F	45	PPMS	4 Years		3.5
1.1	F	59	No	—	Spouse	—
1.2	F	66	No	—	Sibling	—
1.3	M	47	No	—	Spouse	—

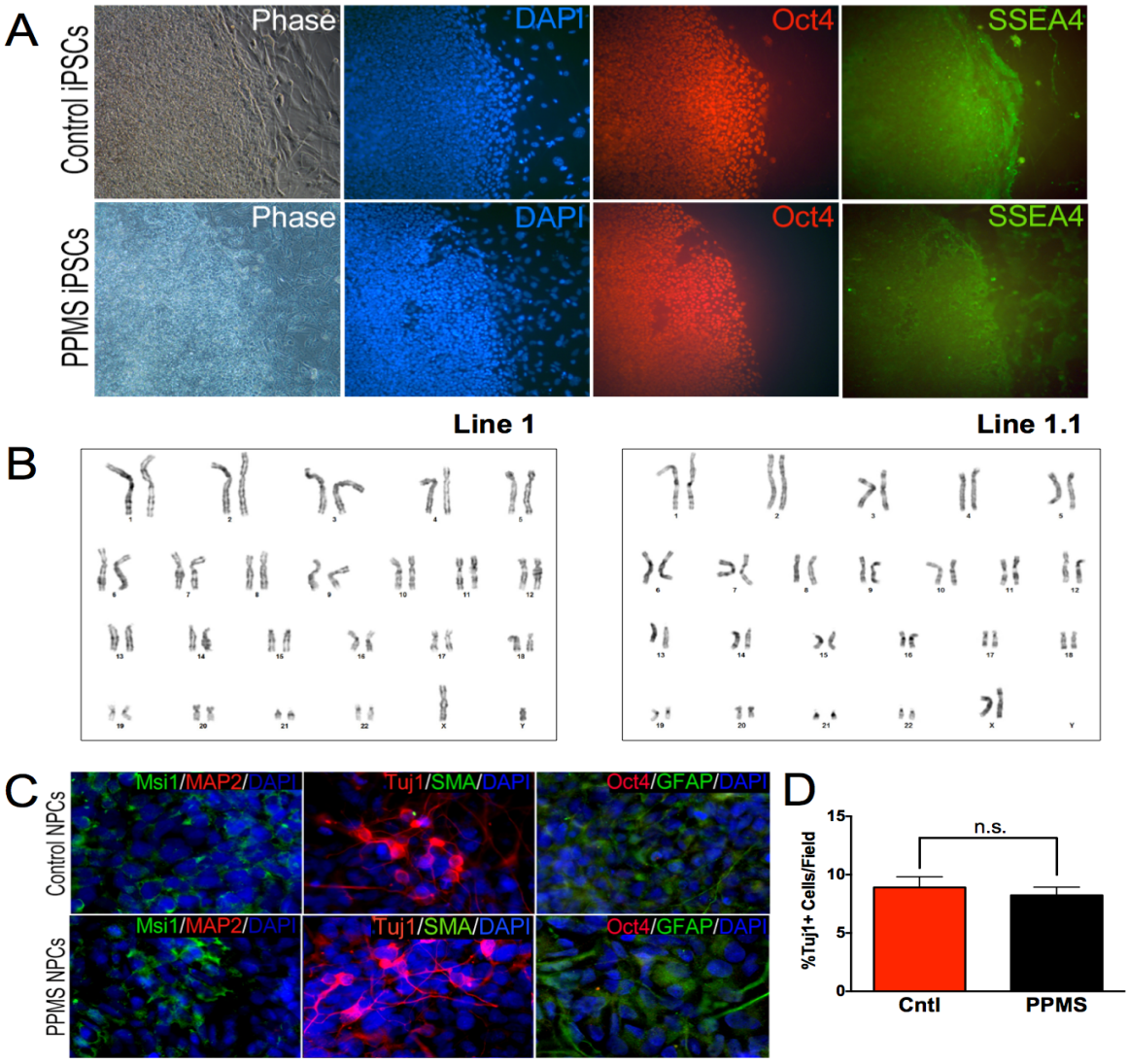
VIII. Supplement

Supplemental Methods

iPS Cell Lines developed from PPMS and Control Blood Samples and NPC Differentiation. The stemness and genomic integrity of all iPS cell lines was validated by immunocytochemical analyses for Oct4 and SSEA4 and karyotyping analyses. Results indicated successful and uniform reprogramming across all lines with colonies from each line exhibiting similar morphology (Supp. Fig. 4.4A). Karyotyping of both iPSC line 1 (PPMS) and line 1.1 (control) also revealed no chromosomal anomalies (Supp. Fig. 4.4B). NPC differentiation was induced by treating the iPS cells with neurobasal media supplemented with noggin (250 ng/mL) for 10 days to promote formation of neural rosettes [347]. NPC differentiation was validated by assaying expression of Musashi-1 (Msi1), βIII-tubulin (Tuj1), and GFAP (Supp. Fig. 4.4C). NPCs from disease and control lines exhibited similar proportions of mature cell types: with the control line expressing an average of 8.9 ± 0.9% positive Tuj1 cells and the PPMS line an average of 8.2 ± 0.7% positive Tuj1 cells (P = 0.5731; Supp. Fig. 4.4D). A lack of microtubule-associated protein 2 (MAP2), a post-mitotic neuronal marker indicated no terminally differentiated neurons in the NPC cultures, and a lack of Olig2 expression indicated no oligodendrocyte lineage cells, which supported the characteristic early proliferative nature of these cultures. Validation of the restricted potential of these NPC cultures was also validated by the lack of smooth muscle actin (SMA) or Oct4 staining, indicating that there was no contamination by either mesodermal or earlier stage stem cells in these cultures. These data indicate successful reprogramming to iPS cells and equivalent differentiation of both control and PPMS cells to NPCs.

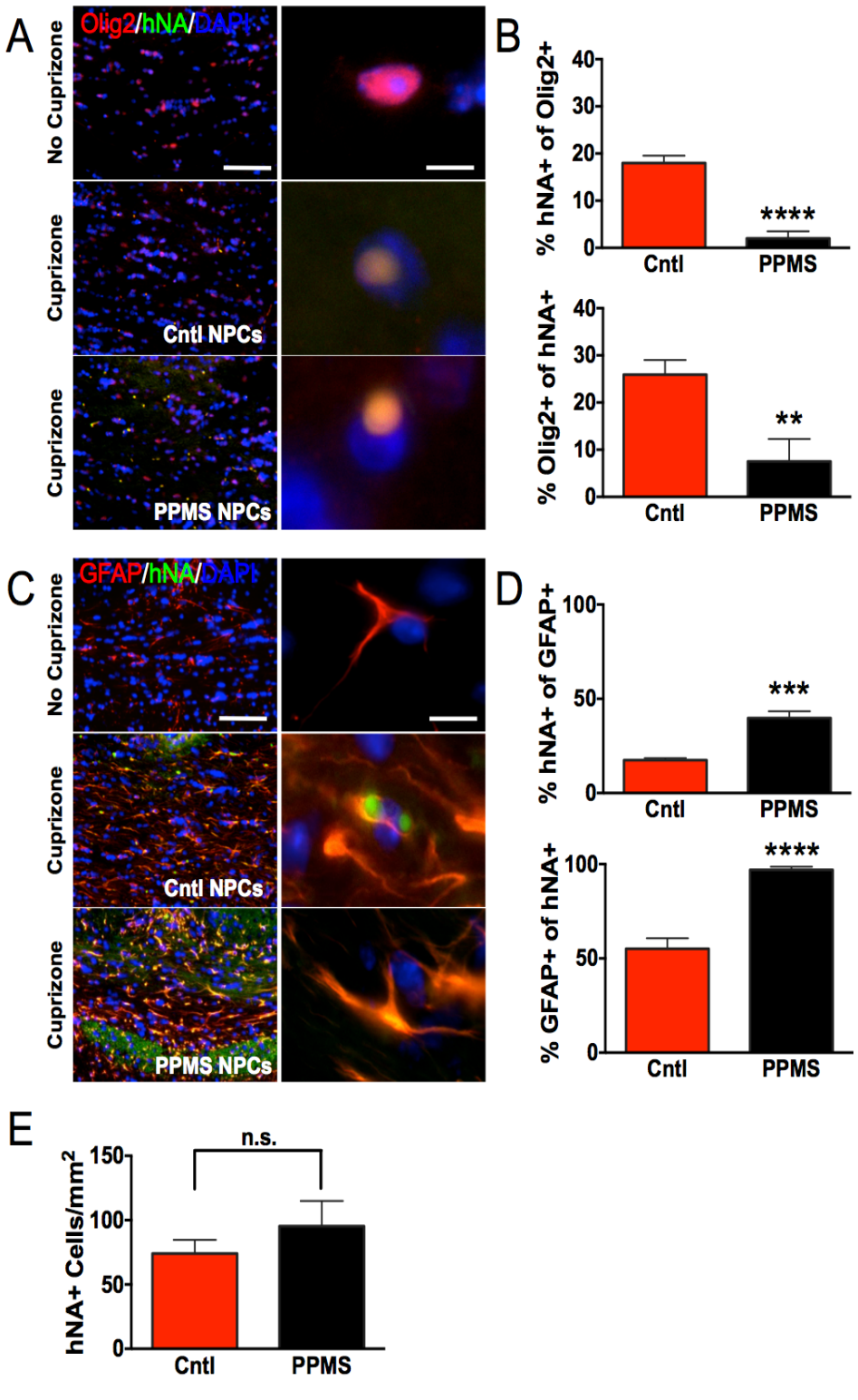
Immunohistochemistry. Brain sections were deparaffinized, and immunohistochemical staining was performed as previously described using the following primary antibodies [336, 348]: Olig2 (1:250; Millipore, Temecula, CA, USA), MBP (1:500; Millipore, Temecula, CA, USA), and human nuclear antigen (1:150; Millipore, Temecula, CA, USA). Coronal brain tissue sections (between

1.10 mm rostral to bregma and 1.7 mm caudal to bregma) were used for all analyses. After deparrafinization of the mouse sections, they were either stained using GFAP and human nuclear antigen or using Olig2 and human nuclear antigen (hNA). These immunostained sections were examined at 20x magnification and images of the medial CC were collected using identical settings. Images were viewed and analyzed using computer-assisted image analysis software on an Olympus IX71 microscope. For each section the total number of cells positive for GFAP or human nuclear antigen were counted, and the cells positive for Olig2 or human nuclear antigen were counted. In addition, the cells that were double labeled were also counted. For each animal in the treatment groups, three sections were analyzed.



Supplemental Figure 4.4

Supplemental Figure 4.4. Control and PPMS iPS cells and NPCs exhibit equivalent pluripotency and differentiation markers *in vitro*. *(A)* Immunocytochemical analyses determined that iPS cells from both lines expressed markers Oct4 and SSEA4. No morphological differences were observed between the two lines. *(B)* Karyotype of PPMS line 1 and control line 1.1 indicates no chromosomal abnormalities in the iPSCs (Table 4.1). *(C)* Between D16-D21 of NPC differentiation immunocytochemistry was performed to assay for expression of markers indicative of neural differentiation, Msi1, MAP2, Tuj1, and GFAP. No positive staining for SMA or Oct4 indicated no mesodermal or earlier stage stem cells in these cultures. We also did not detect any Olig2 staining in either control or PPMS cultures, which indicated no differences in differentiation potential of the NPCs. *(D)* Both lines of NPCs expressed the same amount of Tuj1 indicating no differences in maturity (n = 6 separate coverslips/line). Representative figures in panels A, B, and C are control line 1.1 and PPMS line 1. Values are means ± SEM.



Supplemental Figure 4.5

Supplemental Figure 4.5. Cell fate analysis of transplanted human cells in cuprizone-treated mice. Analysis of human cell fates in the corpus callosum of cuprizone-treated mice administered control NPCs (Line 1.1) or PPMS NPCs (Line 1). *(A, B)* Analysis of oligodendrocyte lineage (Olig2+) cells in these treatment groups identified a significantly lower percentage of Olig2+ staining that colocalized with human nuclear antigen (hNA+) staining in the PPMS NPC injected mice. First scale bar, 200 μm; second scale bar 15 μm. (*n* = 187 hNA+ cells in control NPC injected mice, *n* = 196 hNA+ cells in PPMS NPC injected mice). *(C, D)* Analysis of hNA+ immunostaining with GFAP+ (astrocytes) determined a higher proportion of hNA+/GFAP+ cells in PPMS NPC-treated mice than in control NPC-treated mice. (*n* = 257 hNA+ cells in control NPC injected mice, *n* = 376 hNA+ cells in PPMS NPC injected mice). *(E)* Quantification of hNA+ cells in medial corpus callosum of mice that had been injected with either control NPCs or PPMS NPCs (as described). There were no significant differences in number of hNA+ cells per mm^2 (P = 0.3753). Error bars represent SEM. (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Student's *t* test).

Chapter 5: Cellular Senescence in Neural Progenitor Cells Underlies Myelin Defect in Progressive Multiple Sclerosis

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I. Abstract

Cellular senescence is a form of adaptive cellular physiology associated with aging. Cellular senescence causes a pro-inflammatory cellular phenotype that impairs tissue regeneration, has been linked to stress, and is implicated in several human neurodegenerative diseases. We had previously determined that neural progenitor cells (NPCs) derived from primary progressive multiple sclerosis (PPMS) patient induced pluripotent stem (iPS) cell lines failed to promote oligodendrocyte progenitor cell (OPC) maturation whereas NPCs from age-matched control cell lines did so efficiently. Herein, we report that expression of hallmarks of cellular senescence were identified in Sox2+ NPCs within white matter lesions of human progressive MS autopsy brain tissues and PPMS patient iPS-derived NPCs. Expression of cellular senescence genes in PPMS NPCs was found to be reversible by treatment with rapamycin which then enhanced PPMS NPC support for oligodendrocyte differentiation. A proteomic analysis of the PPMS NPC secretome identified high mobility group box-1 (HMGB1), which was found to be a senescence-associated inhibitor of oligodendrocyte differentiation. Transcriptome analysis of OPCs revealed that senescent NPCs induced expression of epigenetic regulators mediated by extracellular HMGB1. Lastly, we determined that NPCs are a source of elevated HMGB1 in human white matter lesions. Based on these data, we conclude that cellular senescence contributes to altered NPC functions in demyelinated lesions in MS. Moreover, these data implicate cellular aging and senescence as a process that contributes to remyelination failure in progressive MS which may impact how this disease is modeled and inform development of future myelin regeneration strategies.

Significance Statement

Nicaise et al. identify cellular senescence occurs in neural progenitor cells (NPCs) from primary progressive multiple sclerosis (PPMS). In this study, senescent NPCs were identified within demyelinated white matter lesions in progressive MS autopsy tissue, and iPS-derived NPCs from

PPMS patients were found to express cellular senescence markers compared to age-matched control NPCs. Reversal of this cellular senescence phenotype, by treatment with rapamycin, restored PPMS NPC-mediated support for oligodendrocyte maturation. Proteomic and histological analyses identify senescent NPCs in PPMS as a source of HMGB1 which limits maturation and promotes transcriptomic changes in oligodendrocytes. These findings provide the first evidence that cellular senescence is an active process in PPMS that may contribute to limited remyelination in disease.

II. Introduction

Cellular senescence is a component of the aging process that is increasingly recognized as an important pathophysiological mechanism associated with a variety of neurodegenerative human diseases [216, 349]. Cellular senescence is distinct from cellular quiescence in that senescent cells develop a unique level of cellular activity that often exerts a pro-inflammatory paracrine effect onto surrounding cells which can impair or alter tissue function, termed the senescence-associated secretory phenotype (SASP) [269, 279]. Cellular senescence is linked with increased activity of mTOR (mammalian target of rapamycin) [350]. Rapamycin has been found to decrease the production of the SASP through inhibition of mTORC1 [279], which has enabled studies on cellular plasticity under cellular senescence and the contribution of SASP factors to tissue and organ function. One notable influence of cellular senescence is on stem cells where senescence has been shown to reduce trophic support and lessen the regenerative capacity which can contribute to impaired healing and tissue atrophy in aging and disease [211, 351, 352].

Progressive forms of multiple sclerosis (PMS) represent a form of this demyelinating disease of the central nervous system (CNS) without relapses or remissions. The unabating disability in PMS is attributed to demyelination with minimal remyelination [80]. Promoting brain repair represents a potential strategy to restore neurologic function in PMS patients [327]. However, the success of potential remyelinating therapies relies on endogenous oligodendrocyte progenitor cells (OPCs) to differentiate into myelinating oligodendrocytes (OLs) [353]. Therefore understanding what limits the potential of endogenous OPCs for remyelination will be required to realize remyelination as a therapeutic opportunity for regeneration.

Studies analyzing white matter lesion pathology in PMS brain tissues have identified immature, premyelinating OPCs in association with neural progenitor cells (NPCs) [319]. NPCs have been found to be capable of secreting factors that are anti-inflammatory and support remyelination [146], therefore both these cell types could be viewed as capable of promoting

regeneration; yet OPC differentiation *in vivo* is limited and mostly arrested resulting in chronic demyelination [23, 80, 81, 318, 319]. We have recently identified that NPCs differentiated from iPS cells derived from primary progressive MS (PPMS) patients were less able to provide neuroprotection to myelin injury or support OPC differentiation *in vitro* [111]. Importantly, OPCs differentiated from PPMS patient iPS cell lines are equally capable of maturing into myelin-forming oligodendrocytes [157], demonstrating that OPCs in this disease have potential that may be limited by a disease phenotype in NPCs.

In this study, we report new evidence for cellular senescence in NPCs in PMS and provide functional evidence that this is a reversible process which is responsible for limiting oligodendrocyte maturation. These data suggest that while MS is not a disease of aging, at the cellular level, it may be an aging-related disease.

III. Materials and Methods

Human Tissue Immunohistochemistry. Unfixed frozen post-mortem tissue was provided by UK Multiple Sclerosis Tissue Bank via a UK prospective donor scheme with full ethical approval (MREC/02/2/39) and the MRC Sudden Death Brain Bank (ethical approval LREC16/ES/0084) (Table 5.1). Colorimetric staining was used to detect labeling in human tissue. 10 μm sections were fixed in 4% PFA and antigen retrieval was performed by microwaving sections in Vector H-3300 antigen unmasking solution (Vector Laboratories, Peterborough, UK). Slides were blocked with Bloxall^{TM} blocking solution (Vector) then further blocking was performed with 2.5% horse serum (Vector). Sections were incubated with primary antibodies HMGB1 (1:500 mouse, Biolegend) or p16^{Ink4a} (1:500 mouse, Thermo Fisher Scientific, Paisley, UK) in antibody diluent (Spring Bioscience, Abcam, Cambridge, UK) overnight at 4°C. Sections were incubated with Impress^{TM} HRP anti-mouse then colorized with DAB (Vector). Sections were washed and blocked in 2.5% horse serum (Vector) then incubated with Sox2 (1:500 rabbit, Reprocell, Glasgow, UK) overnight at 4°C. Sections were incubated with Impress^{TM} AP anti-rabbit and colorized with Vector Blue alkaline phosphatase then mounted in Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Sections were imaged on a Zeiss Axio Scan.Z1 and processed using Qupath software [354]. Four areas measuring 0.37 mm^2 each were counted within both the lesion and NAWM in MS tissue and white matter of control tissue.

iPS Cell and NPC Culture Derivation. Induced pluripotent stem (iPS) cells and NPCs were generated as described previously [111], and iPSC lines from the Fossati lab were derived as described previously [157] (Table 5.2). Cell colonies were passaged manually when needed, and NPC passage number was kept consistent between lines, similar by at least 5 passages for all experiments described.

Isolation of Rat Oligodendrocyte Progenitor Cells (OPCs) and Treatment with NPC Conditioned Media (CM). OPCs were obtained from the cerebral cortices of neonatal rat pups (postnatal day 0-2), and were plated on poly-L-ornithine coated coverslips, as previously described [336]. OPCs were cultured in varying conditioned mediums for 48 hours. CM was taken from the NPCs after 48 hours on 80% confluent cells, at least 4 days after passage, and immediately spun down to remove debris then frozen at -80°C. The conditioned media experiments were performed in quadruplicate technical replicates and repeated in triplicate using separately developed cultures (i.e. unique biological replicates). For the HMGB1 blocking experiments, CM was collected then incubated with 1 µg/mL of function blocking αHMGB1 antibody (BioLegend, San Diego, CA), or the appropriate isotype control (IgG) for 1 hour at 37°C on a shaker. The treated CM was then applied to OPCs for 48 hours, and then differentiation was assayed. For recombinant HMGB1 treatment, OPCs were grown in differentiation media (neurobasal media [Thermo Fisher, Waltham, MA], 2% B27, 2 mM L-glutamine, and 10 ng/mL T3) and treated with recombinant human HMGB1 (R&D Systems, Minneapolis, MN) every 24 hours for 48 hours, for a total of two doses. Doses consisted of: 0.5, 5, 50, 500, and 5000 ng/mL. OPCs were then fixed and differentiation assayed. Oligodendrocytes on coverslips were fixed and stained for Olig2 (1:500; Millipore, Temecula, CA, USA) and MBP (1:500; Millipore). For all experiments five fields of view at 20x using identical image capture settings were assessed by an experimenter blinded to treatments (Olympus IX71, CellSens Software, Olympus, Center Valley, PA). For analysis of OPC differentiation, all Olig2+ cells and MBP+ cells were counted and the percentage of MBP+ cells calculated. Data is represented as the percent of MBP+/Olig2+ cells relative to the control condition set to 100%.

Neural Progenitor Cell Treatments. NPCs were treated with rapamycin (12.5 nM, daily for 48 hrs) once cells became 80% confluent. After treatment, conditioned media was collected, and

cells collected in either TriReagent (Millipore Sigma, Burlington, MA) for RNA isolation, RIPA buffer for protein isolation, or fixed (4% paraformaldehyde) for immunocytochemistry.

Immunocytochemistry (ICC). NPCs were plated on laminin-coated coverslips (50 µg/mL, Sigma Aldrich), and fixed using 4% paraformaldehyde (PFA). Following fixation they were permeabilized using triton-X100 (0.5%, Millipore Sigma). Cells were then stained using the following primary antibodies overnight: $p16^{Ink4a}$ (1:500; ThermoFisher, Rockford, IL, USA) and Sox2 (1:250; Stemgent, San Diego, CA, USA), and then appropriate secondary antibodies were applied.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR). Total RNA was isolated from NPCs plated on laminin as described previously [348]**,** and converted into complementary DNA (cDNA) via reverse transcription (iScript cDNA synthesis kit, BioRad, Hercules, MA), according to the manufacturer's protocol. Synthesized cDNA samples were amplified for qRT-PCR using primer pairs for human $p16^{Ink4a}$ (F: 5' GAAGGTCCCTCAGACATCCCC 3'; Rev: 5' CCCTGTAGGACCTTCGGTGAC 3'), human p53 (F: 5' CCCCTCCTGGCCCCTGTCATCTTC 3'; Rev: 5' GCAGCGCCTCACAACCTCCGTCAT 3', human HMGB1 (F: 5' ACATCCAAAATCTTGATCAGTTA 3'; Rev: 5' AGGACAGACTTTCAAAATGTTT 3'), and human β-actin (F: 5' GGACTTCGAGCAAGAGATGG 3'; Rev: 5' AGCACTGTGTTGGCGTACAG 3') using the SsoAdvanced Universal SYBR Green Supermix (BioRad) according to the manufacturer's protocol. The PCR cycle conditions were 1 cycle at 95°C for 2 min, 40 cycles of 95°C for 5 s, and 40 cycles at 60°C for 30 s run on a BioRad CFX96 Touch Real-Time PCR Detection System. Relative expression of mRNA was calculated relative to β-actin using comparative cycle threshold analysis.

Senescence β-Galactosidase Staining. NPCs were plated onto laminin-coated coverglass at equal densities (2 million cells/35 mm well), and β-galactosidase staining was performed

according to the manufacturer's instructions (Cell Signaling Technology, Danvers, MA; Supp. Fig. 5.6). Images were captured at 10x using identical phase contrast settings (Olympus IX71, CellSens Software, Olympus).

Immunoblots. Cells were lysed in RIPA buffer, and protein content determined by BCA, and 20 µg of protein was separated by electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in tris-buffered saline (1% tween-20) (TBST) + 5% bovine serum albumin (BSA), incubated overnight with primary antibodies: phosoho-mTOR (Ser2448) (Cell Signaling Technology, Danvers, MA) and GAPDH (Cell Signaling), washed in TBST, and incubated with the appropriate HRP-conjugated secondary antibody for an hour. Proteins were visualized by chemiluminescence.

Mass Spectrometry. Conditioned media from iPS-derived NPCs from either non-disease control, PPMS, or PPMS lines treated with rapamycin were collected and sent to the Mass Spectrometry and Proteomics Keck Laboratory at Yale University where LC-MS/MS analysis was performed was performed on a Thermo Scientific Q Exactive Plus with a Waters nanoAcquity UPLC system, using a Waters Symmetry® C18 180 µm x 20 mm trap column and an ACQUITY UPLC PST (BEH) C18 nanoACQUITY Column 1.7 µm, 75 µm x 250 mm (37ºC) for peptide separation. Trapping was done at 5 µl/min, 97% Buffer A (100% water, 0.1% formic acid) for 3 min. Peptide separation was performed at 330 nl/min with Buffer A: 100% water, 0.1% formic acid and Buffer B: 100% acetonitrile, 0.1% formic acid. A linear gradient (90 minutes) was run with 3% buffer B at initial conditions; 5% B at 1 minute; 35% B at 50 minutes; 50% B at 60 minutes; 90% B at 65-70; and back to initial conditions at 71 minutes. MS was acquired in profile mode over the 300-1,700 m/z range using 1 microscan; 70,000 resolution; AGC target of 3E6; and a full max ion time of 45 ms. MS/MS was acquired in centroid mode using 1 microscan; 17,500 resolution; AGC target of 1E5; full max IT of 100 ms; 1.7 m/z isolation window; normalized collision energy of 28;

and 200-2,000 m/z scan range. Up to 20 MS/MS were collected per MS scan on species with an intensity threshold of 2E4, charge states 2-6, peptide match preferred, and dynamic exclusion set to 20 seconds. Data was viewed using Scaffold Viewer (Proteome Software, Portland, Oregon). Analysis of protein lists was performed using the DAVID bioinformatics database [355, 356], and the percentage of proteins from the varying mediums associated with disease was analyzed using the Genetic Association Database disease class. Percentages shown in the pie graph are the percent proteins involved in that disease category over total proteins.

RNA sequencing. OPCs were plated out and treated with NPC CM from control, PPMS, and PPMS + αHMGB1 CM as described above. After 48 hours the OPCs were collected in Trizol, and RNA was isolated as described previously [348]. RNA was given to Jackson Laboratories (Farmington, CT) where 1 µg of total RNA was processed using the TruSeq RNA Library Preparation Kit v2 (Illumina, RS-122-2001) according to the manufacturer's instructions. The protocol starts from using oligo-dT attached magnetic beads to purify the poly-A containing mRNA molecules. The purified mRNA was fragmented and reversed to first stand cDNA. Then second strand cDNA was synthesized. After end repair and a single 'A' nucleotide was added to the 3'ends, cDNA was ligated to its indexing adapter. Four cycles of PCR were used to enrich the adapter ligated DNA fragments. Following bead purification, libraries were quantified and equally pooled together. The pooled libraries were sequenced on the Illumina HiSeq 4000 platform using a 75 base paired-end protocol and sequenced to a depth of approximately 40M reads per library. The RNA-seq samples were processed using the in-house pipeline at The Jackson Laboratory. The reference for rat (version 6.0.91) was obtained from Ensemble. Alignment estimation of gene expression levels using the EM algorithm for single-ended and paired-end read data was performed using RSEM (package version 1.3.0); default settings were used for alignment. Data quality control was performed using Picard (version 1.95) and qualimap (version 2.2.1). Qualimap output was utilized to examine the alignment data and to detect potential biases in mapping data;

this was computed using two analyses types: 1) BAMQC; 2) RNA-seq QC within the tool. BAMQC provides basic alignment statistics (e.g. coverage, GC content). RNA-seq QC provided quality control metrics, bias estimations, transcript coverage and 5'-3' bias computation. Analyses of aligned reads were performed using the edgeR (v3.30.7) and Biobase (2.38.0) packages in R (v3.4.3). Following normalization, read counts were filtered to include genes with counts per million (CPM) greater than one in at least two samples. The quantile-adjusted conditional maximum likelihood (qCML) method was used to determine differentially expressed genes between libraries as follows: 1) control and PPMS; 2) control and PPMS + αHMGB1; 3) PPMS and PPMS + αHMGB1. Genes with FDRs < 0.05 were sorted by gene ontology using Gene Ontology Consortium and PANTHER analysis to get varying clusters, including: myelin, oxidative stress, epigenetics, and senescence. All heatmaps were made in RStudio (V. 1.1.419) using packages "gplots" and "RColorBrewer" and organized by gene log fold change.

Statistical Analysis. Data were analyzed by Student's t-test or one-way ANOVA with Tukey's multiple comparisons test, where appropriate and as indicated, using GraphPad Prism version 7 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com). Differences were considered significant when $P < 0.05$. Data are presented as mean ± SEM.

IV. Results

Cellular Senescence is Present in NPCs of Progressive MS Brain Tissue and in iPS-derived NPCs from PPMS Patients.

Age is recognized as an irreversible process that limits tissue regeneration and impairs CNS remyelination [206, 284]. We hypothesized that the process of cellular aging called cellular senescence may contribute to differences in support for myelination previously reported by NPCs derived from PPMS and non-disease control iPS cell lines [111]. Human autopsy brain tissue samples from confirmed progressive MS patients and age matched controls were immunostained for the NPC marker Sox2 along with $p16^{Ink4a}$, a cyclin-dependent kinase inhibitor and an established marker of senescence [357]. Within the progressive MS brain we found there to be a significant increase in the number of Sox2+ NPCs in white matter lesions when compared with either normal appearing white matter (NAWM) or white matter of control brain samples (Table 5.1; Fig. 5.1A, B). This increase in NPCs within demyelinated lesions was consistent with previously reported observations [118]. However, when co-labeled with $p16^{ink4a}$, we identified that the majority of Sox2+ NPCs within the progressive MS brain expressed this senescence marker (Fig. 5.1A, C). These data indicated the presence of elevated cellular senescence in NPCs in the progressive MS brain.

To further characterize cellular senescence in PPMS NPCs, and to interrogate a functional role for this aging process in human NPCs, we differentiated NPCs from iPS cell lines of PPMS patients and age-matched control donors. We first determined whether the expression of the lysosomal enzyme β-galactosidase, also a marker of cellular senescence [358], was present in the iPS-derived NPCs (Supp. Fig. 5.6A). Elevated activity of senescence associated β-gal (SA-β gal) in PPMS NPCs was observed while little to no activity was detected in any NPCs from non-disease control iPS cell lines (Fig. 5.1D, E, Supp. Fig. 5.6A demonstrates quantification methodology). We then examined by quantitative PCR (qPCR) the expression of $p16^{Ink4a}$ a well-known tumor suppressor gene also known as a marker of cellular senescence [209, 359].

Significantly higher mRNA expression of $p16^{Ink4a}$ was measured in PPMS NPCs when compared to control NPCs (Fig. 5.1F) [360]. Since cellular senescence is known to be associated with cell cycle arrest [212], we performed cell cycle analysis and examined the proportion of NPCs in G0/G1, G2/M, and S phase. We found no significant differences between control and PPMS NPCs in terms of cell cycle indicating that evidence of senescence in PPMS NPCs was not accountable to replicative senescence (Supp. Fig. 5.6B). Collectively, these independent markers of cellular senescence identified increased senescence in PPMS NPCs when compared to the age-matched control NPCs, even though the chronological age of the human donors in both groups did not differ (Table 5.2). We also considered whether these changes may relate to differentiation. To test this, we assayed $p16^{ink4a}$ expression in the undifferentiated iPS cell state for each iPS cell line. Interestingly, we found no differences in $p16^{Ink4a}$ mRNA expression among the different cell lines (Supp. Fig. 5.7), suggesting that these early passage iPS cell lines adopted a cellular senescent phenotype only when differentiated.

Reversal of Cellular Senescence in NPCs Promotes OPC Maturation.

The identification of cellular senescence in PPMS NPCs led us to hypothesize that this intrinsic process may underlie the failure of PPMS NPCs to support OPC maturation we had previously reported [111]. Given that a senescent phenotype also contributes to a secretory phenotype, we selected to affect cellular senescence in PPMS NPCs using rapamycin, which would also block the senescence-associated secretory phenotype, or SASP. We first confirmed that treatment of PPMS NPCs with rapamycin (12.5 nM, 48 h) resulted in decreased mTOR phosphorylation in PPMS NPCs, as determined by Western blotting (Fig 5.2A, B). We then assayed expression of senescent markers. This brief rapamycin treatment of PPMS NPCs decreased $p16^{Ink4a}$ mRNA and protein expression assayed by qPCR and immunocytochemistry, respectively (Fig. 5.2C, D). SA-β gal staining also decreased in intensity to that observed in non-diseased control NPC following

rapamycin treatment (Fig. 5.2E). These findings demonstrated that rapamycin effectively reduced expression of key cellular senescence markers $p16^{ink4a}$ and SA-β gal, in PPMS NPCs.

We next determined whether rapamycin affected the potential for PPMS NPCs to support OPC differentiation. To test this, we collected conditioned media (CM) from PPMS NPC cultures that had either been treated with rapamycin or vehicle, and these media were separately applied to oligodendrocyte progenitor cell cultures (OPCs). Consistent with our previous work [111], we found that CM from PPMS NPCs did not promote the differentiation of OPCs, when compared to control NPC CM, and did not alter OPC numbers (Fig. 5.2F, G, H). In contrast, OPCs grown in CM from rapamycin-treated PPMS NPCs were found to differentiate into MBP+ oligodendrocyte at a rate comparable to OPCs grown under control NPC CM conditions (Fig. 5.2 H). Treatment of the control NPCs with rapamycin did not negatively affect their ability to promote OL maturation via CM (Fig. 5.2G, H). This effect of rapamycin on human NPCs contrasts with previous studies examining the direct effects of mTOR signaling on OPCs [361, 362], indicating that rapamycin was not transferred to the OPCs in the CM. However, to verify that this was the case, we also tested whether carry over of rapamycin could have independently promoted OPC differentiation in OPCs grown in PPMS CM. OPCs were treated directly with the same concentration of rapamycin used to treat the NPCs (12.5 nM) and we found no increase in differentiation (Supp. Fig. 5.8). This was consistent with previous work that had shown direct treatment of OPCs with rapamycin may actually inhibit OPC differentiation [363]. Hence, reversing senescence in PPMS NPCs using rapamycin was found to provide not only a change in senescence marker expression but also result in a significant improvement in PPMS NPC function reflected by enhanced OPC differentiation. Importantly, treatment of OPCs under any of these media conditions tested did not result in differences in cell death, as the number of Olig2+ cells did not differ among any of the experimental treatment conditions (Fig. 5.2H).

Proteomic Analysis of Neural Progenitor Cell Secretome Identified HMGB1 as a Potent Inhibitor of OL Differentiation.

To identify what factor(s) in the NPC conditioned media were responsible for impaired OPC differentiation, we performed mass spectrometry (LC-MS/MS) on the CM from control NPCs, PPMS NPCs, and also rapamycin-treated PPMS NPCs to identify and compare peptides from each of these conditions (Fig. 5.3A). For this analysis we exploited the heterogeneity of the human donors to provide higher stringency to the comparisons where only factors found in all samples in a treatment group were considered valid targets. From these proteomic analyses we identified several factors uniquely expressed by PPMS NPCs. Many of these peptides represented secreted proteins previously known to be associated with cellular senescence, including heat shock proteins 90 and 60, DJ-1, and HMGB1 (high mobility group box 1) (Fig. 5.3A). Importantly, these factors were not found to be produced by either the control NPCs or when PPMS NPCs had been treated with rapamycin (Fig. 5.3A). Ontology analysis of the identified peptides using the DAVID bioinformatics database revealed that 14.5% of proteins secreted by PPMS NPCs were associated with “aging”. This “aging” profile was reduced to only 4.8% of peptides identified from PPMS NPC that had been treated with rapamycin, which represents a decrease of 67%. No aging ontology was identified from the secretome of control NPCs (Fig. 5.3B). These findings lend further support to cellular senescence as a feature of NPCs in PPMS and rapamycin as a pharmacological means to affect the SASP of NPCs.

Of the candidate proteins secreted by PPMS NPCs, we focused our attention on HMGB1 for several reasons: it is produced at higher levels in demyelinated lesions in MS brain tissues [237, 364], produced at higher levels by senescent cells [364], and has been implicated as a mediator of neuroinflammation [365]. We evaluated *HMGB1* gene expression and found significantly increased mRNA expression in PPMS NPCs which was also down-regulated by rapamycin treatment (Fig. 5.3C). To address the contribution of extracellular HMGB1 to impaired OPC differentiation, we repeated the OPC differentiation experiment using CM from PPMS NPCs

but added a well characterized function blocking antibody to inhibit HMGB1 (αHMGB1) (Fig. 5.3D). Maturation of OLs in the αHMGB1-treated PPMS CM was significantly higher when compared to OPCs grown in CM with serotype-matched IgG antisera (control) (Fig. 5.3E). Blocking HMGB1 was not found to affect either Olig2+ cell proliferation or cell death (Fig. 5.3F). To test whether extracellular HMGB1 was acting directly as an inhibitor of OPC differentiation we applied recombinant human (rh)HMGB1 to cultures of differentiating OPCs and measured the proportion of OL maturation (Fig. 5.3G). Extracellular rhHMGB1 had a concentration-dependent effect on reducing OPC differentiation *in vitro* (Fig. 5.3H). Lastly, we determined whether NPCs could be a source of HMGB1 within demyelinated lesions in human MS brain tissues. Immunohistochemistry identified a significantly increased proportion of Sox2+/HMGB1+ NPCs in demyelinated white matter lesions when compared with NAWM (Fig. 5.3I, J). These data provide the first direct evidence that extracellular HMGB1 produced by senescent NPCs can act to directly suppress OPC maturation.

HMGB1 Mediates Senescence-related Transcriptomic Changes in OPCs

To better understand how cellular senescence of NPCs and their production of extracellular HMGB1 affects the potential for OPCs to mature into MBP+ OLs with myelinating potential, we performed transcriptomic analyses of OPCs. Specifically, collected cultures of primary OPCs that had been grown in CM from either PPMS NPCs, non-disease control NPCs or CM from PPMS NPCs that had been treated with αHMGB1 antisera. OPCs were grown for 48 hours and then collected for RNA sequencing analyses (i.e. RNAseq, Fig. 5.4A). Genes were filtered for a false discovery rate of <0.05. Substantial transcriptome changes were seen in the OPCs treated with either control CM, PPMS CM, or PPMS + αHMGB1 (Fig. 5.4B, C, D). Out of all genes filtered, Cd99l2 and Wdr54 were found to be some of the most upregulated genes in the OPCs treated with PPMS CM compared to the OPCs treated with control CM (Fig. 5.4B). Cd99l2 has been found to allow for the entrance of leukocytes into the CNS in the experimental autoimmune

encephalomyelitis (EAE) mouse model of MS, and when knocked out leukocyte entry is inhibited and disease ameliorated [366]. Treatment of the PPMS CM with αHMGB1 did not decrease Wdr54 and Cd99l2 to levels found in OPCs treated with control CM (Fig. 5.4C). Treatment of the OPCs with PPMS + αHMGB1 CM did increase expression of Map2k3 (Fig. 5.4B), which was found to be significantly decreased in OPCs treated with PPMS CM (Fig. 5.4D). Map2k3, a dual specificity kinase, has been found to be associated with a resolution in inflammation [367]. Our data therefore implicate HMGB1 in perpetuating inflammation in OPCs.

Our first step in curating these data was to identify differentially expressed genes (DEGs), which defined several notable ontological differences between the treatment groups. Senescent cells also are known to induce senescence through the secretion of the SASP as a paracrine effect, so we examined markers of senescence in the OPCs. Overall, we found an increase in senescence-associated genes, such as *Mmp2*, $p16^{Ink4a}$ (*Cdkn2a*), and *Igfbp2* when OPCs were treated with PPMS CM, which was reduced close to normal in PPMS CM containing HMGB1 blocking antibody (Fig. 5.4E). We also investigated expression of epigenetic modifiers since epigenetics plays a pivotal role in determining the potential for OPC differentiation [138]. We found that PPMS CM significantly induced expression of numerous epigenetic regulators in OPCs (Fig. 5.4F). Interestingly, when HMGB1 was functionally blocked from the PPMS NPC CM most of these changes in epigenetic-associated genes were normalized to levels observed in control NPC CM (Fig. 5.4F). These RNA sequencing data indicate that the expression and production of HMGB1 by NPCs plays an important role influencing the potential for OPCs to mature by modulating gene expression. Moreover, these differences in senescence-associated and epigenetic modifier genes in OPCs demonstrate that it is likely locally produced factors from NPCs within demyelinated lesions that contribute to the microenvironment that determine OPC fate. These observations provide a novel and compelling link between the presence of senescent NPCs in demyelinated lesions in progressive MS and ascribe a deleterious role for cellular

senescence in NPCs which we have functionally characterized using iPS cells from PPMS patients.

V. Discussion and Conclusions

Our data provide a new perspective on the pathophysiology of demyelinating lesions in MS. We have identified cellular senescence in Sox2+ NPCs which influence, through paracrine activity, the maturation of OPCs with myelinating potential. We determined that cellular senescence is potentially reversible, or amenable to therapeutic intervention, using rapamycin as a prototypic compound. We also determined that senescent NPCs secrete extracellular HMGB1 which leads to altered gene expression and impaired maturation of OPCs (Fig. 5.5). Age is recognized as a process that limits the myelinating potential of the CNS [284]. Although MS is not typically considered a disease of aging, because it is generally diagnosed in early-to-mid adulthood, the functional impact of cellular senescence in NPCs we report here may indicate that advanced cellular aging is an important component of this disease, particularly in the progressive phase.

Neural progenitor cells are of particular interest because they have been found within demyelinated lesions, along with astrocytes and microglia [23, 81, 317]. As our data support, NPCs are found in greater numbers within the lesion area, when compared to NAWM and control white matter, where they contribute to regulation of OPC maturation and myelin regeneration [318, 319]. Data would suggest that NPCs are active participants in the lesion environment and could secrete regenerative, anti-inflammatory, and promyelinating factors [146, 149, 319]. However, our data would suggest that in progressive MS, endogenous NPCs are or become senescent and their participation in the lesion is pro-inflammatory and actively suppresses OPC maturation through the secretion of factors (the SASP), HMGB1 in particular. These findings contribute to our understanding of the lesion microenvironment and may explain why immature OPCs within lesions fail to remyelinate in progressive MS.

Our data demonstrate that NPCs from patients with PPMS display key hallmarks of senescence, including SA-β gal staining and increased $p16^{Ink4a}$. $p16^{Ink4a}$ is a cyclin dependent kinase inhibitor that has become a hallmark for identifying senescent cells [228]. $p16^{Ink4a}$ acts

through the retinoblastoma pathway, which inhibits the action of cyclin dependent kinases [368]. Up-regulation of $p16^{Ink4a}$ results in chromatin reorganization, which leads to changes in genes related to inflammation and oxidative damage, including COX1 and COX2 enzymes, as well as other inflammatory mediators [368, 369]. We have shown that rapamycin effectively reduced the expression of $p16^{Ink4a}$ in PPMS NPCs and this was reflected in a functional change in the NPC secretome by enhanced OPC maturation. Recent studies using mice have demonstrated that targeted ablation of $p16^{Ink4a}$ positive cells can be used as a strategy to study cellular senescence. Induced deletion of $p16^{Ink4a}$ from senescent cells has been shown to extend lifespan and reverse age-related functional decline in organs such as kidneys and pancreas [227, 228]. Similarly, targeting of cellular senescence by deletion of $p16^{Ink4a}$ positive cells has also been found to mitigate accelerated aging in diseases such as progeria, and more recently in glia in a model of AD; demonstrating $p16^{Ink4a}$ has a central role in the functional effects of cellular senescence [224, 227-232]. Interestingly, the methylation pattern of $p16^{Ink4a}$ has been proposed to be an epigenetic risk factor in the development of MS [320]. Our study is the first to implicate cellular senescence as a feature of NPCs in MS. As current mouse models of induced demyelination do not reflect the unique aging characteristics of the human disease, the utility of these models to explore the effects of cellular senescence on demyelination is limited. This emphasizes the utility of the approach we have taken here – the use of patient iPS cells and study of human autopsy tissues - to address the question of cellular aging and demyelination.

Whether NPCs in PPMS acquire senescence because of the disease, or are somehow predisposed to developing senescence which promotes the disease is presently unclear. It has been shown that the DNA damage response (DDR) is strongly linked to senescence in other cell types where mitochondrial dysfunction has also been implicated [237]. Cells with damaged mitochondria more easily acquire a senescent phenotype and secrete factors such as CCL27, TNF-α, and HMGB1 [237]. We found HMGB1 only in CM from PPMS cells, and after treatment with rapamycin HMGB1 secretion was abrogated. Extracellular HMGB1 acts as a cytokine and

generates inflammatory responses such as the production of additional cytokines and chemokines, continuing the cycle of chronic inflammation [365]. Interestingly, extracellular HMGB1 has also been found to act as a chemokine specifically for neural progenitor cells, which may further facilitate the senescent microenvironment in the lesion area [370]. HMGB1 can also be secreted by activated astrocytes and microglia in lesion areas, this has been proposed to help recruit NPCs to facilitate repair [371]. However, as our data indicate, if NPCs become senescent and begin secreting HMGB1, this impairs OPC differentiation, and thus is deleterious. Our findings demonstrate HMGB1 as a potent suppressor of oligodendrocyte differentiation. We found a significant increase in NPCs expressing HMGB1 in lesions from progressive MS autopsy tissue. A report has also found increased HMGB1 in MS lesions associated with an elevated expression of HMGB1-binding receptors [372], making it a potential target for progressive MS treatment.

Senescence is not only induced from aging (termed replicative senescence), where cells wear out their capability of replicating, but also from constant inundation of inflammation, which leads to stress-induced senescence [237, 373-375]. The majority of senescent cells release a heterogeneous secretory profile, SASP, that consists of pro-inflammatory cytokines that can disrupt tissue microenvironments and molecules that have paracrine effects on surrounding cells [212, 213, 237, 376]. Changes in senescent gene expression depend on the cell type, which makes the SASP unique to the specific cell of interest [213]. In performing RNA sequencing on the rat OPCs treated with PPMS CM we found an induction of senescence genes. When cross-referencing upregulated genes found in OPCs treated with PPMS CM with those in genome-wide association studies (GWAS) performed on patients with MS we identified VCAM1, STAT3, and MYB(AHI1) [377]. Interestingly, these genes found in MS GWAS studies have also been found to be implicated in senescence [378-380]. Senescent stem cells have been shown to secrete IL-6 which maintains neighboring stem cells in a pluripotent state [245, 381]. Therefore, senescent PPMS NPCs may be affecting other stem cells in the lesion microenvironment by preventing differentiation through the secretion of the SASP. This concept has been termed the “senescence-

stem lock model" [211]. This model demonstrates how senescent cells reduce tissue regeneration and promote aging.

Persistent DNA damage to cells, which has been reported in human MS lesion tissue [288], triggers senescence, and with that, the secretion of the SASP [321]. Consistent inflammation leads to general epigenetic modifications that can leave cells hypersensitive to DNA damage, and in turn, senescent [321, 322]. The chronic demyelination seen in progressive MS patients triggers the brain microenvironment, and the blood, to show persistent signs of oxidative stress and inflammation [288, 323]. This continuous inflammation induces epigenetic changes reflected in the patient NPCs making them more susceptible to DNA damage, and therefore senescent. We found that when HMGB1 was blocked in PPMS CM the effect of the SASP on the OPCs was greatly diminished, and this effect was reflected in changes in the OPC transcriptome. In particular, we identified unique gene expression profiles in OPCs when exposed to CMs from senescent (PPMS) NPCs. For example, we observed a significant up-regulation of both senescence and epigenetic-associated genes in OPCs treated with the PPMS CM. Importantly, we identified extracellular HMGB1 as a mediator of these transcriptional changes associated with impaired or reduced OPC differentiation. Our data demonstrated that HMGB1 had a significant impact on the expression of key epigenetic regulators in OPCs, such as helicase [382, 383], and the histones: H2afz, H2afv, and H2afx [384]. Previous research has targeted HMGB1 as a modifier of gene promoters and repressors involved in pro-inflammation [385]. Overall epigenetic modification of OPCs has been identified as a critical determinant in promoting differentiation and eventual remyelination in mouse models [125, 386, 387].

The cause of progressive MS is not known. Based on these data we hypothesize that cellular senescence in NPCs secrete factors which impair the regenerative potential of the lesion microenvironment. Current treatments for MS suppress inflammation and block access of immune cells into the CNS, yet this does not fully prevent demyelination and axonal degeneration [388]. Additionally, current disease modifying therapies for MS do not benefit patients with progressive

MS [328]. Therefore, targeting and stimulating OPC differentiation has important therapeutic potential, but candidate remyelinating therapies do not take into account the lesion environment. The efficacy of these candidate drugs relies on the differentiation of resident OPCs within the lesioned microenvironment yet if senescent NPCs are not also addressed then therapeutic efficacy may be reduced. Rapamycin is a known immunosuppressant and is currently used to prevent organ transplant rejection, but it has never been investigated in treating PPMS. Interestingly, rapamycin had been tested in clinical trials (NCT00095329, NCT00228397) for patients with relapse-remitting MS, but progressive MS patients were not included and thus the potential of its anti-senescence impact on PPMS patients remain untested. At present, it is unclear whether cellular senescence is present in other types of MS, such as RRMS, but future study would be expected to extend our findings herein and address salient questions regarding whether cellular senescence is found in all forms or MS or perhaps only in progressive forms of MS, and whether predisposition to developing cellular senescence is a risk factor for developing this disease.

In summary, our identification of cellular senescence within the MS brain provides a mechanism by which aberrant cellular aging silently subverts natural aging by impairing OPCs and promoting chronic demyelination. Targeting cellular senescence in NPCs, as a means by which to unlock the differentiation potential of endogenous OPCs, may represent a novel therapeutic approach to promote remyelination in MS.

VI. Acknowledgements and Author Contributions

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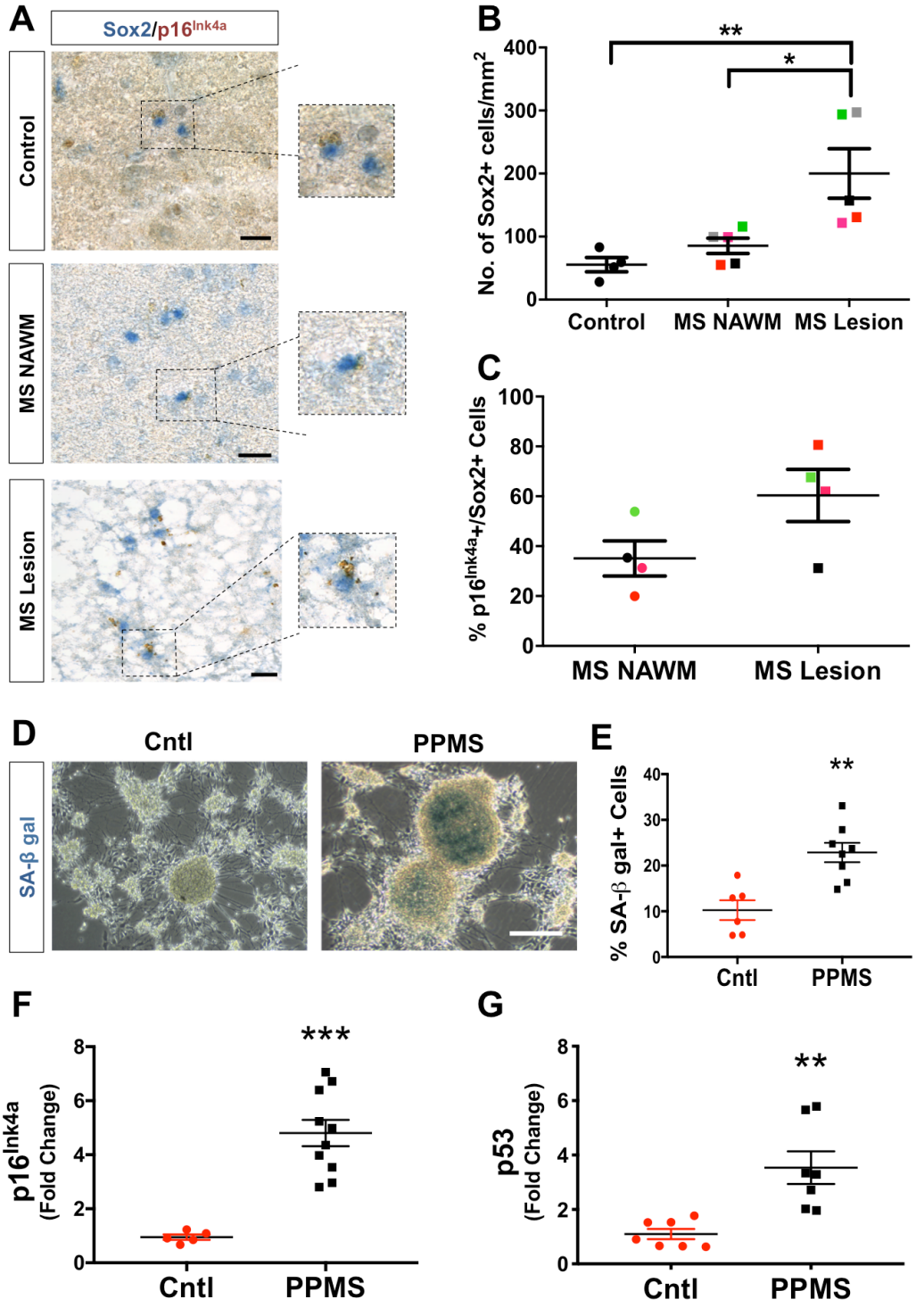
A.M.N. designed and performed experiments, analyzed data, and wrote the manuscript; C.M.W. performed experiments and assisted with analyses; C.P. performed analysis of RNAseq data; L.W. performed human tissue staining and analysis; P.R. provided expertise on RNAseq data analysis and presentation of data, and edited the manuscript; V.F. provided iPSC lines and expertise on stem cell studies with advice on experimental design, and edited the manuscript. A.W. performed analysis of immunohistological staining of human brain tissue samples, experimental design and edited the manuscript; S.J.C. conceived and designed the study, analyzed data, coordinated data collection, wrote and edited the manuscript with A.M.N and with input from all authors.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Control
MS NAWM
MS Lesion
B
No. of Sox2+ cells/mm2
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300
200
100
0
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*
Control
MS NAWM
MS Lesion
C
% p16Ink4a+/Sox2+ Cells
100
80
60
40
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Cntl
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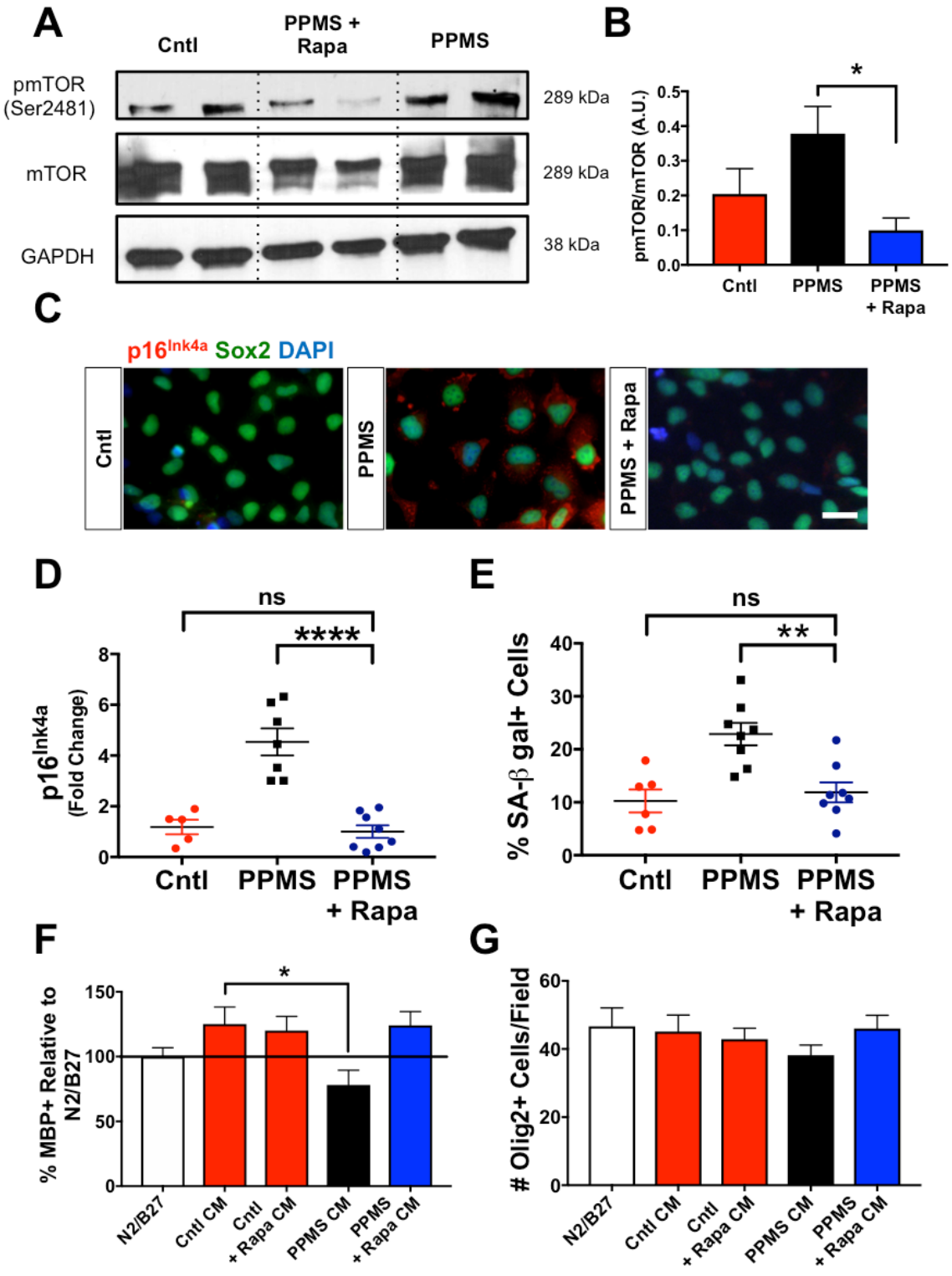
Cntl
PPMS

Figure 5.1

Figure 5.1. Senescent markers identify neural progenitor cells within demyelinated lesion of human progressive MS brain tissue and in NPCs derived from PPMS patients. *(A)* Human brain tissue from the frontal lobe, subcortical white matter was stained for Sox2 (NPC marker) and $p16^{Ink4a}$ (senescence marker) in control and progressive MS patients neurospecimens. Representative image from control is (60 y.o. female), and representative images from MS NAWM and MS lesion (69 y.o. female; SPMS disease duration of 40 years). MS lesion is from a female, 46 year old patient with SPMS and a disease duration of 25 years. Arrows point to cells double positive for Sox2 and $p16^{Ink4a}$. (Scale bar, 20 μm.) *(B)* Increase in total number of Sox2 positive cells in MS lesion compared to control and MS NAWM area. Individual points are individual patients and matching colors between MS NAWM and MS lesion samples represent the same patient (*, $P < 0.05$, ** $P < 0.01$, ANOVA, Tukey's). *(C)* Percent of Sox2+ NPCs co-labeling with $p16^{Ink4a}$ in both MS NAWM and MS lesion areas in same patients identified by color coding ($P = 0.1644$, two-tailed t test). *(D)* Senescence-associated β galactosidase (SA-β gal) staining in control NPC and PPMS NPC cultures revealed LacZ activity in PPMS NPC cultures. Figures show representative lines 3.1 and 3. (Scale bar, 400 μm.) *(E)* Quantification of SA-β gal staining in control and PPMS NPCs show an increase in LacZ in PPMS cultures (**, $P < 0.01$, t test). *(F, G)* $p16^{Ink4a}$ and p53 mRNA expression by qPCR in control NPC and PPMS NPC cultures revealed a significant increase in $p16^{Ink4a}$ and p53 mRNA levels in PPMS NPCs (***, $P < 0.001$, **, $P<0.0022$, unpaired t tests). All qPCR data normalized to control NPC lines. Each data point represents individual sample from varying lines and clones.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Cntl
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PPMS
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mTOR
GAPDH
289 kDa
289 kDa
38 kDa
B
pmTOR/mTOR (A.U.)
0.5
0.4
0.3
0.2
0.1
0.0
*
Cntl
PPMS
PPMS + Rapa
C
p16Ink4a Sox2 DAPI
Cntl
PPMS
PPMS + Rapa
D
p16Ink4a (Fold Change)
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8
6
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2
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Cntl
PPMS
PPMS + Rapa
E
% SA-β gal+ Cells
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40
30
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10
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Cntl
PPMS
PPMS + Rapa
F
% MBP+ Relative to N2/B27
150
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N2/B27
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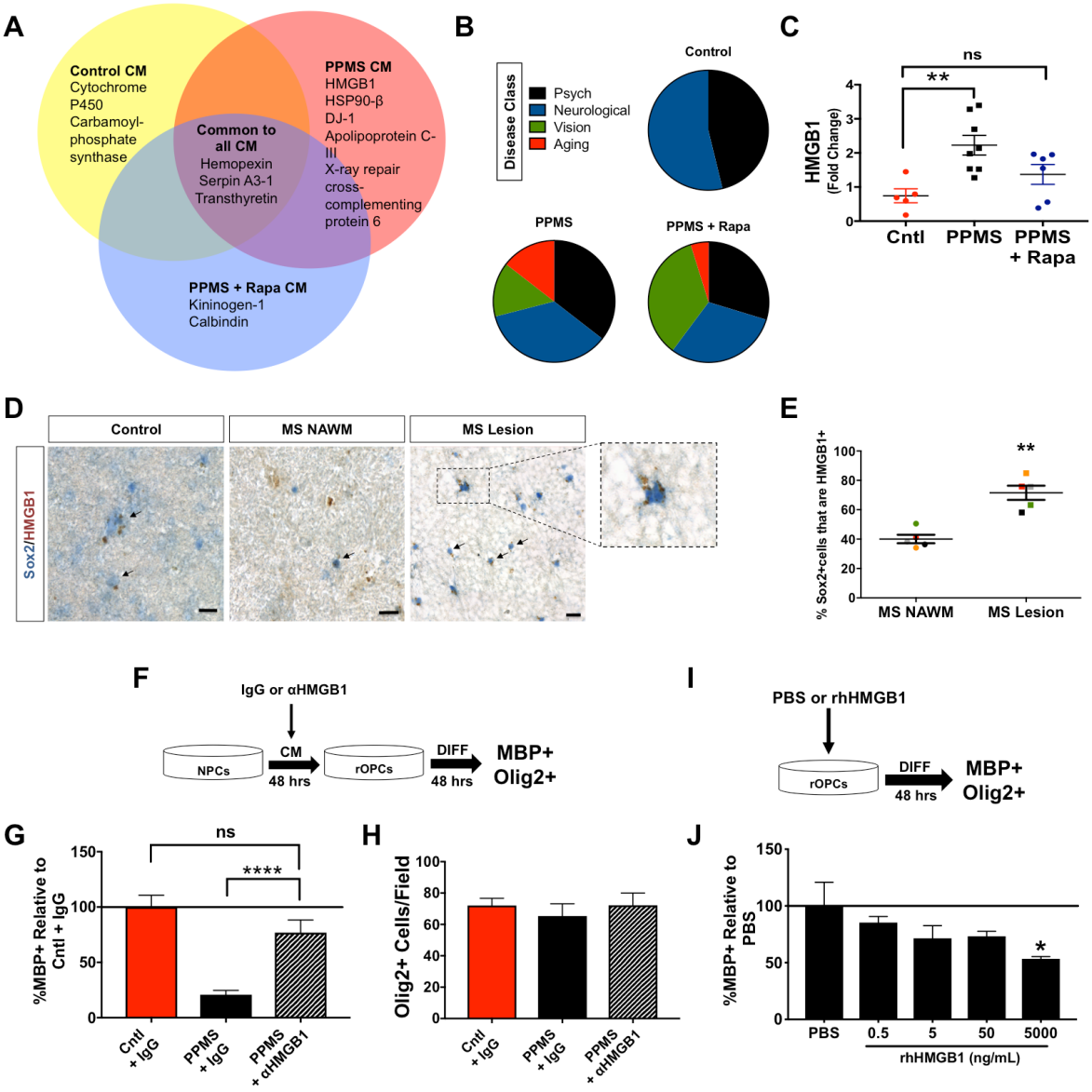
Cntl + Rapa CM
PPMS CM
PPMS + Rapa CM
G
Olig2+ Cells/Field
60
40
20
0
N2/B27
Cntl CM
Cntl + Rapa CM
PPMS CM
PPMS + Rapa CM

Figure 5.2

Figure 5.2. Cellular senescence phenotype in PPMS NPCs is reversed by treatment with rapamycin. *(A)* Western blot of protein lysates from control NPCs, PPMS NPCs, and PPMS NPCs treated with rapamycin for 48 hours probed for phospho-mTOR (Ser2481), mTOR, and GAPDH. *(B)* Densitometry of pmTOR over mTOR shows a significant decrease in pmTOR after rapamycin treatment (*, $P < 0.05$, t test). *(C)* $p16^{Ink4a}$ and Sox2 staining in control, PPMS, and PPMS NPCs treated with rapamycin shows a decrease in $p16^{Ink4a}$ protein expression in the PPMS NPCs. Figure shows representative lines 1 and 1.1. (Scale bar, 50 μm.) *(D)* Decrease in PPMS NPC $p16^{Ink4a}$ mRNA expression after treatment with rapamycin for 48 hours (PPMS vs. PPMS + Rapa [****, $P < 0.0001$], control vs. PPMS [***, $P < 0.001$]; control vs. PPMS + Rapa [$P = 0.6418$], ANOVA, Tukey's). *(E)* Quantification of SA-β gal staining in control NPC, PPMS NPC, and rapamycin-treated PPMS NPCs (PPMS vs. PPMS + Rapa [**, $P < 0.01$], control vs. PPMS [**, $P < 0.01$], control vs. PPMS + Rapa [$P = 0.7483$], ANOVA, Tukey's). All qPCR data normalized to control NPC lines. Each data point represents individual sample from varying lines and clones. *(F)* Quantitative analysis of oligodendrocyte maturation (MBP+/Olig2+) resulting from culture of OPCs in various CM conditions, as indicated, after 48 hrs of treatment. Conditioned media conditions were control NPC CM, control NPC + rapamycin CM, PPMS NPC CM, and PPMS + rapamycin CM. All conditions were quantified relative to differentiation under N2/B27 (standardized control) unconditioned media as set to 100%. (Cntl CM vs. PPMS CM [*, $P < 0.05$], Cntl CM vs. PPMS + rapa CM [$P = 0.9974$], ANOVA, Tukey's). *(G)* Quantification of total Olig2+ cells under each treatment condition showing equivalent densities of cells under all conditions indicating that differences in differentiation were not a result of cellular proliferation or cell death. Each data point represents individual sample from varying lines and clones. Values are means ± SEM.


A
Control CM
Cytochrome P450
Carbamoyl-phosphate synthase
PPMS CM
HMGB1
HSP90-β
DJ-1
Apolipoprotein C-III
X-ray repair cross-complementing protein 6
Common to all CM
Hemopexin
Serpin A3-1
Transthyretin
PPMS + Rapa CM
Kininogen-1
Calbindin
B
Disease Class
Psych
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Vision
Aging
Control
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Cntl
PPMS
PPMS + Rapa
D
Control
MS NAWM
MS Lesion
Sox2/HMGB1
E
% Sox2+cells that are HMGB1+
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MS NAWM
MS Lesion
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IgG or αHMGB1
NPCs
CM
48 hrs
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PPMS + αHMGB1
H
Olig2+ Cells/Field
Cntl + IgG
PPMS + IgG
PPMS + αHMGB1
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%MBP+ Relative to PBS
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PBS
0.5
5
50
5000
rhHMGB1 (ng/mL)

Figure 5.3

Figure 5.3. Proteomic analysis of the NPC secretome and characterization of HMGB1 as a directly acting inhibitor of OPC differentiation. *(A)* Venn diagram of comparison strategy for proteomic data analysis from control CM, PPMS CM, and PPMS + rapa treatment CM. Peptides identified by LC MS/MS from each treatment group. Protein threshold was set at 95% confidence of identification. *(B)* Functional classification of secreted peptides from PPMS NPCs and non-disease control NPCs as determined by the DAVID bioinformatics database (https://david.ncifcrf.gov/). Pie graphs depicting the proportions of peptides identified associated with disease classifications as determined by Genetic Association Database disease class. *(C)* Increased HMGB1 mRNA expression in PPMS NPCs was decreased with rapamycin treatment (Cntl vs. PPMS [**, $P < 0.01$], Cntl vs. PPMS + rapa [$P = 0.4182$], ANOVA, Tukey's). *(D)* Human tissue staining for Sox2 and HMGB1 in control and progressive MS patients in the frontal lobe, subcortical white matter. Representative image for control is from an 82 year old male who died from myelodysplastic syndrome and rheumatoid arthritis, and representative images for MS NAWM and MS lesion is from a female, 69 year old patient with SPMS and a disease duration of 40 years. Arrows point to cells double positive for Sox2 and HMGB1. (Scale bars, 20 μm.) *(E)* Increase in percent of Sox2 positive cells expressing HMGB1 in MS lesion compared to MS NAWM. Individual points are individual patients, and matching colors between MS NAWM and MS lesion samples represent the same patient (** $P < 0.01$, two-tailed t test). Values are means ± SEM. *(F)* Experimental design to test whether functional blocking antisera against HMGB1 (1 μg/mL) in PPMS CM (or IgG control; 1 μg/mL) affected OPC maturation (rOPCs, rat OPCs). *(G)* Quantification of OL maturation in experiment "E". All conditions relative to Cntl + IgG, set to 100% (PPMS + IgG vs. PPMS + αHMGB1 [****, $P < 0.0001$], ANOVA, Tukey's). *(H)* No significant differences in Olig2+ cells were observed from the varying treatments on the OPCs. *(I)* Experimental design to test whether recombinant human (rh)HMGB1 affected OPC maturation (rOPCs, rat OPCs). *(J)* Treatment of OPCs with increasing doses of rhHMGB1 (ng/mL) decreases

OL differentiation after 48 hours in the presence of differentiation media. All conditions relative to PBS, set to 100% (PBS vs. 5000 ng/mL rhHMGB1 [* *P,* < 0.05], ANOVA, Tukey's).

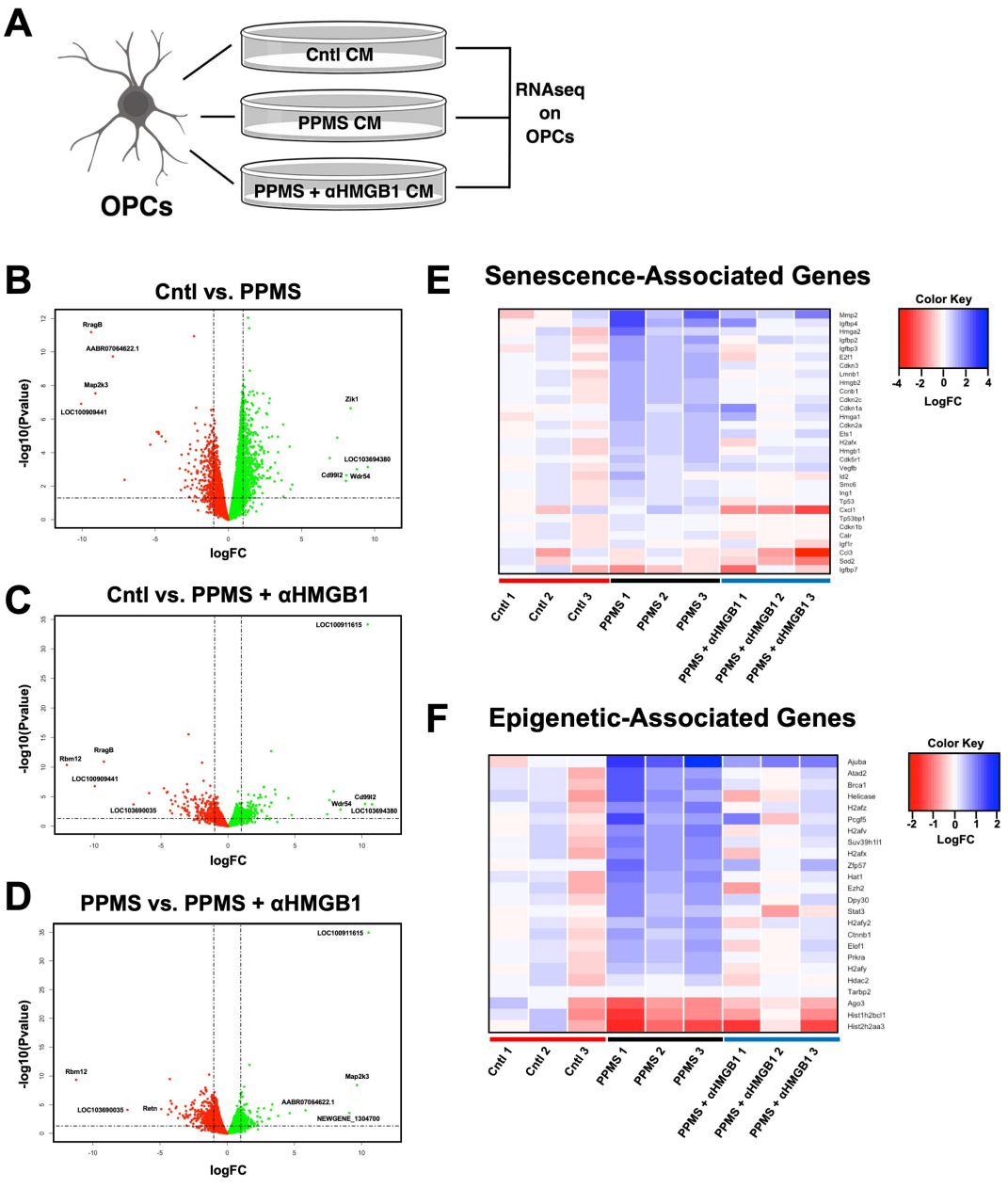

A
Cntl CM
PPMS CM
PPMS + αHMGB1 CM
RNAseq on OPCs
OPCs
B
Cntl vs. PPMS
-log10(Pvalue)
logFC
RragB
AABR07064622.1
Map2k3
LOC100909441
Zik1
LOC103694380
Cd99l2
Wdr54
C
Cntl vs. PPMS + αHMGB1
LOC100911615
RragB
Rbm12
LOC100909441
LOC103690035
Wdr54
Cd99l2
LOC103694380
D
PPMS vs. PPMS + αHMGB1
LOC100911615
Rbm12
Map2k3
LOC103690035
Retn
AABR07064622.1
NEWGENE_1304700
E
Senescence-Associated Genes
Color Key
LogFC
Cntl 1
Cntl 2
Cntl 3
PPMS 1
PPMS 2
PPMS 3
PPMS + αHMGB1 1
PPMS + αHMGB1 2
PPMS + αHMGB1 3
F
Epigenetic-Associated Genes
Color Key
LogFC

Figure 5.4

Figure 5.4. Transcriptomic analysis of OPCs treated with human NPC conditioned media (CM) identifies marked differences in expression of cellular senescence genes (E) and epigenetic regulators (F) regulated by HMGB1. *(A)* OPC RNA was collected after 48 hours of treatment with control CM, PPMS CM, or PPMS + αHMGB1 CM, and RNAseq was performed. Image generated using BioRender. *(B, C, D)* Volcano plots of gene expression changes in the OPC transcriptome after treatment with control CM, PPMS CM, or PPMS + αHMGB1 CM. The x-axis specifies the log of the fold changes (FC) and the y-axis specifics the negative logarithm to the base 10 of the p-vales. Vertical lines reflect a FC of ±2 and the horizontal line reflects a P value of 0.05. *(B)* reflects gene expression changes in control vs. PPMS CM treated OPCs, *(C)* reflects gene expression changes in control vs. PPMS + αHMGB1 CM treated OPCs, and *(D)* reflects gene expression changes in PPMS vs. PPMS αHMGB1 CM treated OPCs. *(E)* Heatmap of expression differences in senescence-associated genes in OPCs after treatment with CM. *(F)* Heatmap of expression differences in epigenetic-associated genes in OPCs after treatment with CM. Three individual control and PPMS lines were selected and are demonstrated individually in the heatmaps. RNA was aligned to the latest version of the rat genome (version 6.0.91). Heatmap for each class of genes, as indicated. Genes with FDRs < 0.05 were sorted by gene ontology using Gene Ontology Consortium and PANTHER analysis to get the varying clusters.

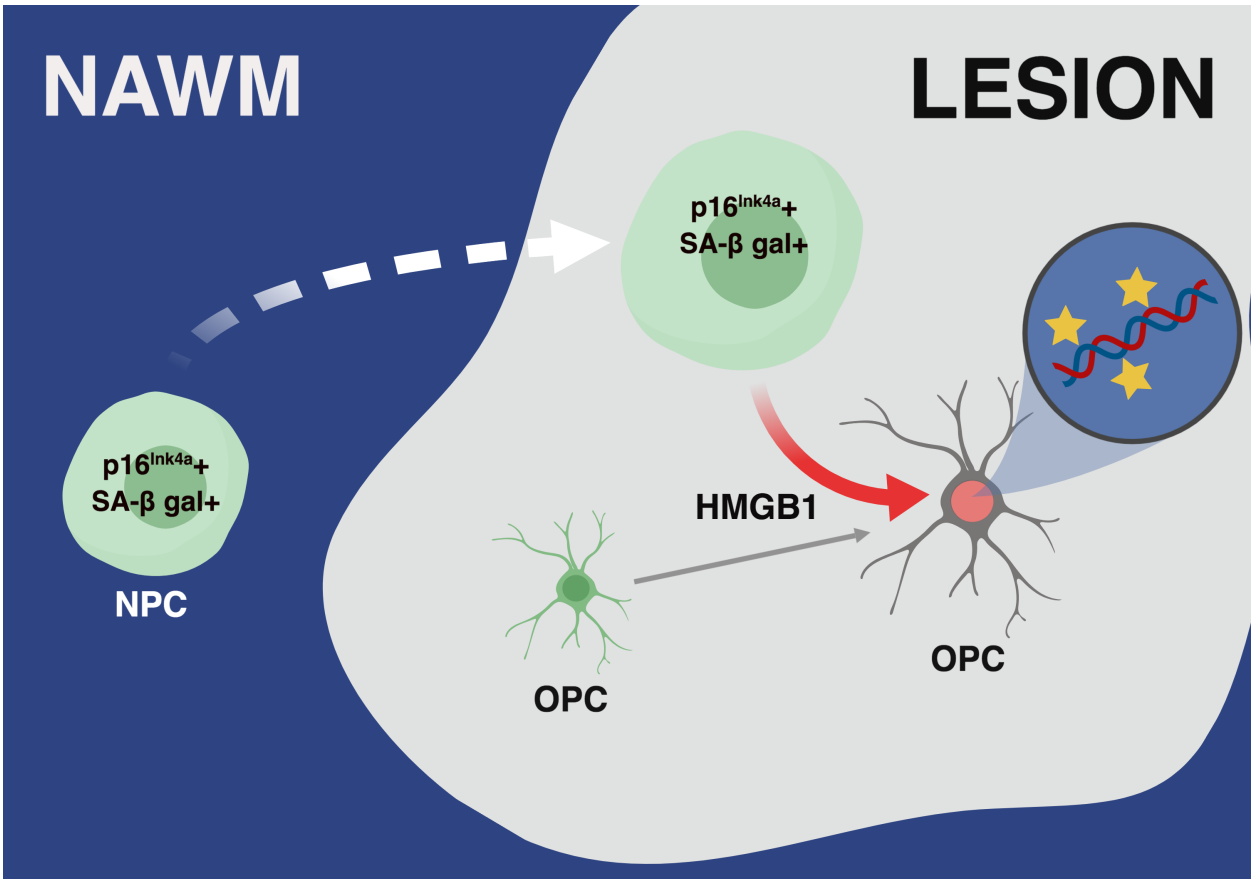

NAWM
LESION
p16Ink4a+
SA-β gal+
p16Ink4a+
SA-β gal+
NPC
HMGB1
OPC
OPC

Figure 5.5

Figure 5.5 Schematic diagram outlining hypothesized impact of senescent NPCs on remyelinating potential in the MS brain. The recruitment and presence of senescent NPCs in demyelinated white matter lesions exert a negative impact on the differentiation of OPCs, also within the demyelinated lesion, by modifying the transcriptional activity of OPCs via secretion of factors in the SASP such as HMGB1. Collectively, this model suggests that cellular senescence in NPCs, either acquired as a result of disease or as a predisposing feature of individuals that develop MS, contributes to the limited potential of OPCs to foster remyelination in the MS brain. Image generated using BioRender.

Table 5.1. Patient information for histology data.

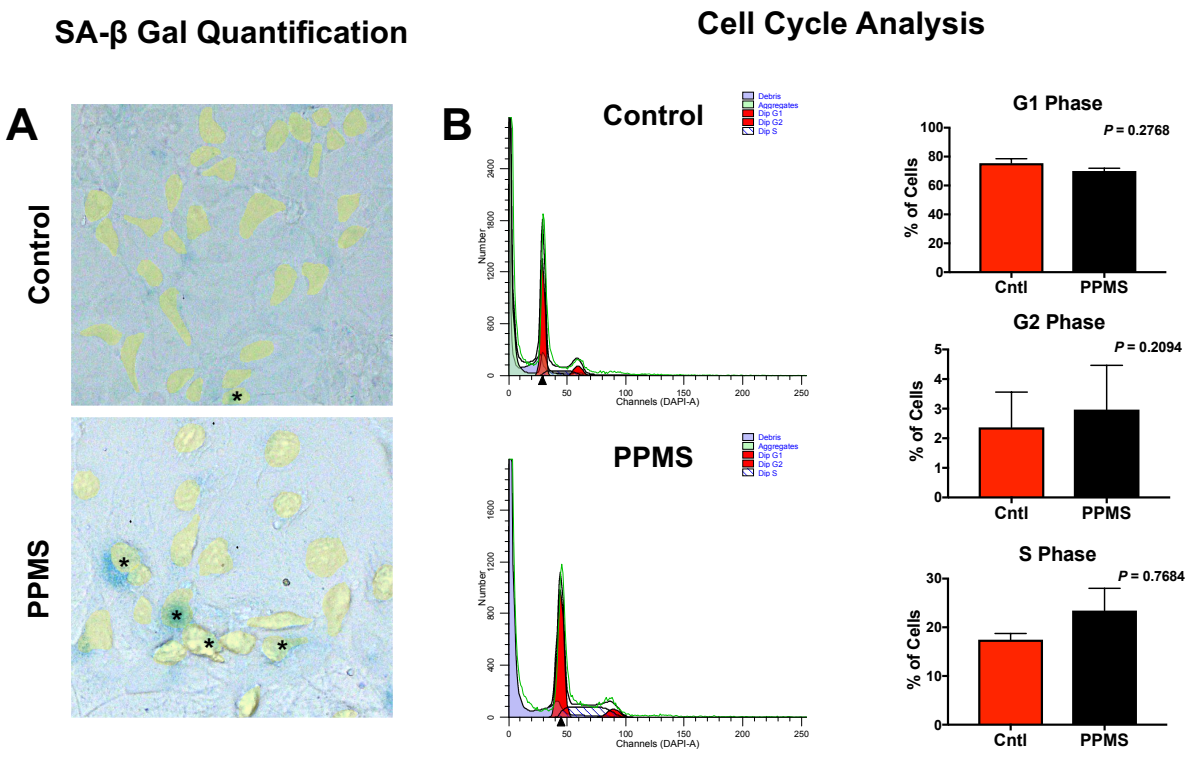
MS Patient	Sex	Age	Disease	Disease Duration (Years)	Post Mortem Delay (Hours)	Graph Color
MS 100	M	46	SPMS	8	7	Gray
MS 121	F	49	SPMS	14	24	Black
MS 207	F	46	SPMS	25	10	Pink
MS 640	F	69	SPMS	40	26	Red
MS 641	F	69	PPMS	19	24	Green
Control Patient	**Sex**	**Age**	**Cause of Death**		**Post Mortem Delay (Hours)**	
SD21/17	M	67	Ischaemic heart disease		68	
SD22/16	M	39	Ischaemic heart disease, coronary artery atherosclerosis		86	
CO25	M	35	Carcinoma of the tongue		22	
CO28	F	60	Ovarian cancer		13	
CO39	M	82	Myelodysplastic syndrome, rheumatoid arthritis		21	

Table 5.2. Induced pluripotent stem cell lines from PPMS patients and controls.

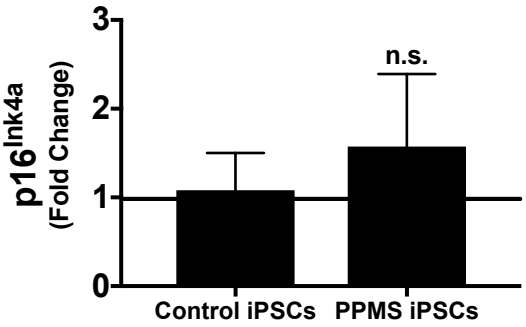
Line	Sex	Age	Disease	Relationship (if known)	Cell Line Source	Cell Origin
1	M	61	PPMS		Crocker	blood
2	F	62	PPMS		Crocker	blood
3	F	45	PPMS		Crocker	blood
104	F	62	PPMS		Fossati	fibroblast
Average Age		**57**				
1.1	F	59		Spouse	Crocker	blood
2.1	F	66		Sibling	Crocker	blood
3.1	M	47		Spouse	Crocker	blood
130	M	52			Fossati	fibroblast
598	M	53			Fossati	fibroblast
Average Age		**55**				

VIII. Supplement

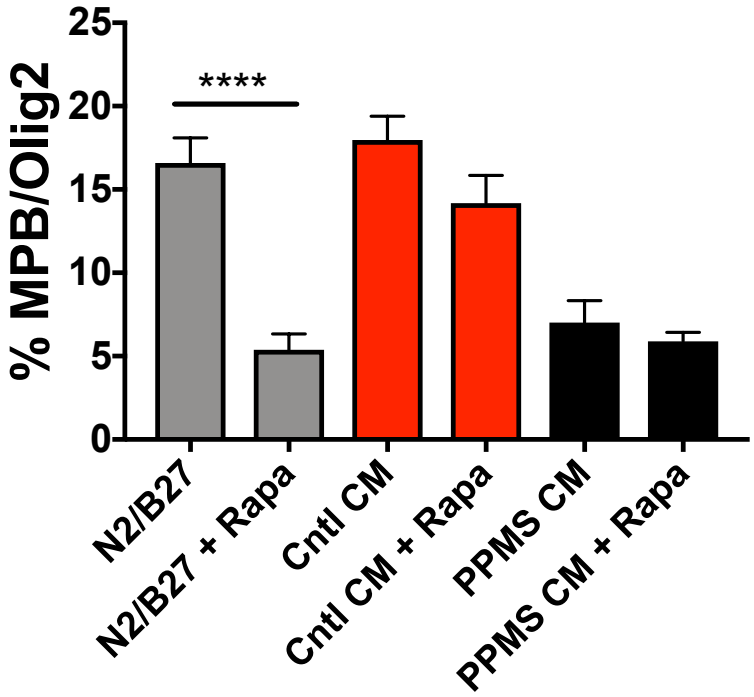
Cell Cycle Analysis. Neural progenitor cells were plated at 750,000 cells per line and then collected and resuspended in PBS 24 hours later. The cells were then incubated in 70% ethanol for 30 minutes at 4°C to fix and permeabilize. The cells were then spun down and washed with PBS. After pelleting the cells they were resuspended in the DAPI stain solution (1 µg/mL DAPI in PBS) and analyzed on an LSR II with the flow rate on slow, and at least 20,000 events were collected. Using FlowJo software the data was analyzed using the Watson (pragmatic) approach to fit Gaussian curves to G1 and G2/M phases and the percent of cells in each phase was calculated.



Supplemental Figure 5.6 Method for quantification of senescence associated β-galactosidase (SA-β gal) staining and cell cycle analysis of NPCs. *(A)* Phase contrast representative images of control and PPMS NPCs with SA-β gal staining. Yellow shading highlights cell bodies and asterisks indicate cells positive for the blue color indicative of SA-β gal staining. *(B)* Representative plots of DAPI cell cycle analysis and graphs of percent of NPCs positive for the G0/G1, G2/M, and S phase. Data demonstrates 4 control NPC lines and 3 PPMS NPC lines. All values are means ± SEM.



Supplemental Figure 5.7. No significant change in $p16^{lnk4a}$ mRNA expression between control and PPMS iPSC lines. All qPCR data normalized to control iPSC lines. Values are means ± SEM. n = 3 iPSC lines / disease condition. Student's t test, *P* = 0.6850.



Supplemental Figure 5.8. Directly added rapamycin does not increase OPC differentiation. Quantitative analysis of mature OLs after direct treatment with media plus rapamycin (12.5 nM) after 48 hours. Direct treatment of rapamycin to OPCs inhibits OL differentiation (****, $P < 0.0001$). Values are means ± SEM.

Chapter 6: Discussion and Future Directions

Since the discovery that induced pluripotent stem cells can be derived from somatic cells they have been used to model a wide range of diseases enabling study of disease-specific processes in a culture dish, testing of new therapeutics, and even have the potential to be used for cell replacement strategies in the future. In my thesis I characterized and analyzed neural progenitor cells derived from iPSCs of patients with primary progressive multiple sclerosis and age-matched controls. My data have demonstrated that NPCs from patients with PPMS display a premature senescent phenotype which impairs their ability to support endogenous remyelination, both *in vitro* and *in vivo*, which was found to be reversed by treatment with rapamycin. My thesis work can be broadly applied to a variety of CNS disorders where age and neurodegeneration is involved, such as Alzheimer's disease (AD) and Parkinson's disease (PD).

I. Genetic Caveats of Modeling Multiple Sclerosis with iPSCs

Classically, iPSCs have been used to model diseases of monogenic origins such as Huntington's disease, caused by the genetic defect of CAG expansion in the huntingtin gene, and familial amyotrophic lateral sclerosis (ALS), with mutations in *SOD1* [389]. iPSCs from these patients retain their somatic genetic traits and are more likely to display cell-autonomous cell defects using stem cell-based modeling [389]. More recently, the use of mutation-defined iPSCs have been used to generate models of neurodegeneration for AD. For example, studies have generated iPSC lines with familial mutations in APP and PSEN-1, known to be involved in AD, not a monogenic disease [198]. Using this type of disease modeling in iPSCs has helped develop drug screening methodologies which can help determine how certain patients will react to various treatments [198].

On the other hand, iPSCs have been recently used to understand non-familial, or sporadic forms, of diseases including sporadic AD and ALS which do not have a defined, specific genetic mutation. Neurons differentiated from iPSCs generated from patients with genetic, familial AD

and those with sporadic AD exhibited higher levels of amyloid-β and phospho-tau compared to non-diseased cells [390]. In another example, astrocytes derived from patients with sporadic ALS were found to be as toxic as those derived from patients with familial, causative mutations [391]. Both these studies have important implications for diseases such as MS, where there is no single mutation known to cause disease. Thus, the potential to model human diseases using the approach of patient iPSCs may help uncover pathological phenotypes that may not have as of yet been identified in patients. Moreover, these data clearly demonstrate that iPSC technologies are not limited to monogenic diseases and have widespread utility for studying complex diseases under defined conditions.

MS does not have defined somatic mutations, and is instead a complex disorder that has been associated with susceptibility genes linked to immunity and environmental factors. Significantly, the current disease modifying therapeutics only work to target the immune component in the disease, which become ineffective once the patient has reached the progressive phase of the disease, or if the patient has always had PPMS. iPSCs have allowed for the modeling of hard to reach cells in the CNS which hold potential for lesion regeneration. Therefore in using patient-specific iPSCs one can model the cells involved in the lesion to understand where regeneration can be targeted. In my thesis I interrogated NPCs derived from iPSCs of patients with PPMS as well as age-matched controls to determine if there were differences in their support of remyelination. I found that NPCs derived from PPMS patients failed to support oligodendrocyte maturation and failed to provide remyelination support *in vivo* due to a premature aging phenotype. Even though PPMS has no specific identified culprit gene and no single genetic mutations are linked to risk for this condition there are clear, reproducible inherent defects in NPCs derived from these patients. Since MS is a disease that involves the interplay between environmental factors and individual genetic susceptibility, epigenetic changes likely underlie the phenotype I have defined using iPSC PPMS NPCs.

The reprogramming process of somatic cells has been suggested to reset genomic methylation and epigenetic modifications. However, it is becoming increasingly clear from reports in the literature that this is not always the case. For example, fibroblasts which have been reprogrammed into iPSCs have been found to differentiate more readily back into fibroblasts [194, 196]. In addition, analysis of DNA methylation in patient-specific iPSC lines has found that epigenetic modifications remained unchanged during the reprogramming process [197]. Patient-derived iPSCs retain epigenetic modifications from the original donor which may explain why the NPCs from PPMS patients demonstrate significant defects. Using iPSCs from patients incorporates environmental risk factors, vitamin D deficiency, smoking, and exposure to the Epstein-Barr virus which have been found to exert epigenetic changes and be involved in the pathogenesis of MS [392]. Hence, accumulating data about reprogramming, including my own, have demonstrated a clear functional distinction between disease and control iPS cells, and support the growing awareness that iPSC reprogramming does not wipe clean the epigenome.

Another way to study various cell types from patients with MS, that could be used to extend my findings, is using the new method of direct reprogramming. It is now possible to take patient fibroblasts and directly reprogram them to the cell type of interest. Currently, direct reprogramming can generate neurons, astrocytes, and NPCs [149, 391, 393, 394]. Direct reprogramming avoids the expensive and laborious process of making iPSCs and has been shown to retain a more direct epigenetic profile of the patient [393]. Using this method may help further parse out epigenetic changes in NPCs from patients with PPMS. Future studies expanding on the epigenetics of NPCs derived from PPMS patients are further warranted to understand the role it may play in the development of defective NPCs.

II. Neural Progenitor Cells and Senescence

The processes of natural aging may be an underlying risk factor for progression in MS, and the cause of failed remyelination. This hypothesis is supported by aging as a natural limit on

remyelination potential in people with progressive MS and rodent models [14, 108, 312, 395]. The data in this thesis demonstrates that NPCs from patients with PPMS fail to foster remyelination as well as neuroprotection due to a premature aging phenotype, senescence. Chronic mitochondrial oxidative stress is a major trigger of cellular senescence [237, 241]. Markers of cellular senescence have been shown to increase in NPCs with normal aging, and are associated with diminished regenerative capacity. Importantly, mitochondria are critical for the development of pro-inflammatory and tissue-damaging senescence [241, 396]. Mitochondria are essential organelles for the generation of cellular energy through signaling and metabolism. Mutations in genes that encode subunits of oxidative phosphorylation complex I, part of the electron transport chain (ETC), have been identified in PPMS, as well as altered enzyme activities, increased free radical production, and oxidative damage [397]. Using MitoSox staining to measure the generation of ROS production, I found a significant increase in the production of ROS in the PPMS NPCs compared to the age-matched controls (Fig. 6.1). This data potentially implicates mitochondrial dysfunction in producing the senescence phenotype in the NPCs from primary progressive MS patients.

The process of aging has been directly characterized by DNA damage and overall genomic instability, which is reflected in somatic DNA mutations in aged human iPSCs [195]. The rate of mitochondrial DNA mutations is at least 10-20 fold higher than that of the nuclear genome, increases with age, and the age-related decline of mitochondrial DNA copy number is associated with late-onset progressive MS [398, 399]. In sequencing patients with progressive MS Ban et al. found a trend in the representation of mitochondrial haplogroup U in those with MS, which is associated with the oxidative phosphorylation complex I [400]. A publication from Tranah et al. found an elevated risk for progressive MS in those with the mitochondrial haplogroups J and T [397]. These single nucleotide polymorphisms within J and T occur in genes that encode subunits of oxidative phosphorylation complex I, which when deficient, is the most frequent cause of bioenergetic dysfunction [401]. Future studies performing mitochondrial sequencing on the NPCs

from PPMS patients could reveal potential underlying genetic abnormalities that lead to the dysfunction of mitochondria, and the ensuing increase of ROS.

In 2016 it was recently discovered that mitochondrial dysfunction can lead directly to cellular senescence and secretion of the SASP [237]. Additionally, absence of mitochondria prevents senescence, closely linking mitochondrial dysfunction to senescence [241]. The factors produced in the SASP of the senescent PPMS NPCs initiated a self-amplifying feedback loop by inducing senescence in healthy OPCs through a paracrine fashion and inhibiting their differentiation into oligodendrocytes. Mitochondrial aging defects have also been found to emerge in directly reprogrammed neurons from aged human fibroblasts, where they showed reduced energy production and increased ROS [393]. My own data have also suggested that iPS-derived NPCs from PPMS patients exhibit evidence of elevated oxidative stress (Fig 6.1). These data insinuate the possibility of mitochondrial defects in the PPMS NPCs that may underlie the senescent phenotype.

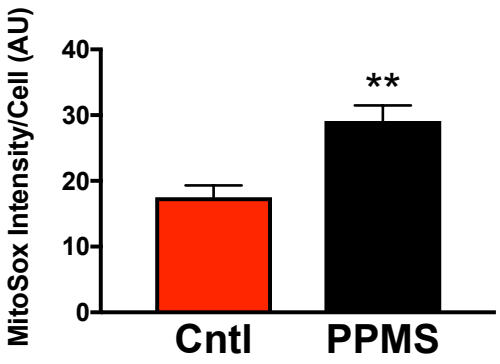


Figure 6.1. NPCs from PPMS patients display increased mitochondrial stress. Increased MitoSox intensity quantified in NPCs from PPMS patients relative to total cell number quantified (Sox2+ cells) (n = 3 lines/group). t-test, **, $P < 0.01$.

In my studies we also applied proteomics to identify HMGB1, high mobility group box 1, as secreted by senescent PPMS NPCs which, in turn, functioned to inhibit OPC differentiation. HMGB1 is a chromatin-associated protein which helps maintain nuclear homeostasis by bending DNA to provide transcription factor access to promoter regions [402]. When secreted it plays a role in inflammation as an alarmin, where it can provoke an innate immune response by binding to cell surface RAGE (receptor for advanced glycation end products) and TLRs (toll-like receptors) [364]. HMGB1 is secreted by senescent cells, and therefore in senescent cells it translocates from the nucleus to the cytoplasm in order to be secreted [364]. Disruption of HMGB1 from the nucleus has been found to induce mitochondrial fragmentation as well as deficits in mitochondrial respiration [403]. Thus, my findings implicate HMGB1 as a putative mechanism contributing to mitochondrial dysfunction which may underlie defects in support of oligodendrocyte growth and maturation evident from the PPMS NPCs.

An important aspect of my studies was the finding that cellular senescence in NPCs was amenable to therapeutic manipulation by rapamycin. Rapamycin is a direct inhibitor of mTOR and my results showed that rapamycin effectively reversed the senescent phenotype in the PPMS NPCs and recovered their ability to support OPC differentiation. Further studies are needed to determine whether rapamycin reversal of cellular senescence is a transient event or may result in a permanent reversal of senescence in the PPMS NPCs. In addition, studies are planned to determine if reversing senescence using rapamycin in iPS-NPCs affects their function when administered to cuprizone treated mice [111]. Importantly, rapamycin has been found to alter mitochondrial dynamics by increasing mitochondrial respiration, decreasing ROS production, and inducing autophagy [404, 405]. In analyzing all facets of the data in this thesis, as well as the literature, including the increased ROS production, senescence, reversal of senescence with rapamycin, and increased mitochondrial DNA mutations in aged progressive MS patients, mitochondrial dysfunction is highly implicated to be the reason for the defects in these cells.

Besides the secretion of HMGB1 that directly inhibits OPC differentiation, it is still unknown exactly how senescent NPCs alter the lesion microenvironment. Overall, the SASP limits tissue regeneration through the secretion of oxidative and pro-inflammatory factors and can induce senescence in surrounding cells [212]. Injected senescent NPCs in the cuprizone mouse model could be inducing senescence in the host tissue, in turn inhibiting endogenous OPC differentiation. Analysis of the mouse lesion sites need to be performed in order to determine if the human cells are conferring senescence locally. In addition, the cuprizone model allows for the study of endogenous remyelination. Determining if senescent NPCs impair endogenous CNS remyelination will help further examine the role of NPCs in the lesion environment. This experiment can be performed by injecting NPCs after cuprizone withdrawal. Treatment of mice with cuprizone for 4 weeks depletes the myelinating oligodendrocytes, but after cuprizone is removed from the diet the resident OPCs will remyelinate [338]. Injection of the PPMS NPCs at this time point will determine if they hinder endogenous remyelination after demyelination. The lysolecithin model could also be used to study remyelination in the presence of senescent NPCs since the key steps in the timeline of the remyelination process is known [305]. Understanding how senescent NPCs affect the lesion microenvironment would potentially open opportunities for identifying new therapeutic targets which would be expected to promote brain repair in progressive MS patients.

III. Do all Subtypes of MS have Senescent NPCs?

Based on the data presented in this thesis there are senescent cells in both primary progressive and secondary progressive forms of the disease. We identified senescent NPCs secreting HMGB1 in lesions from patients with PPMS as well as SPMS. Further characterization of cells from patients with the relapsing-remitting forms is needed. The RRMS form of the disease is much different to the progressive form as it is diagnosed earlier in life, early 20s, and can be treated with immunomodulatory therapies, while the progressive forms have no current

treatments. We hypothesize that due to a shift in age of diagnosis, patients with RRMS will not have a senescent phenotype in the NPCs. By accident, we obtained a single line of iPSCs from a patient with RRMS, line 102. The cells were differentiated into NPCs and assayed for senescence markers. $p16^{Ink4a}$, p53, and HMGB1 were not significantly increased in the NPCs from the RRMS line (Figure 6.2A, B, C). In addition, treatment of OPCs with CM from line 102 did not significantly decrease differentiation into mature OLs (Figure 6.2D). These preliminary data indicate that there are no significant markers of senescence in this line from one RRMS patient, but in order to have a definitive conclusion more lines from varying patients need to be assayed. A more thorough analysis of post-mortem brain tissue from patients with RRMS would also yield more results as to if the NPC population in these tissues are senescent.

Overall these data suggest a previously unrecognized mechanism occurring in progressive forms of MS but not in RRMS, the presence of senescent NPCs inhibiting oligodendrocyte differentiation. The model for our current understanding is that with aging, along with epigenetic factors altering the risk of MS, NPCs become senescent due to the inflammatory lesion environment in progressive MS. Once senescent they no longer can support proper remyelination, and instead secrete the SASP inhibiting regeneration. Further study is needed to explore and better understand this process and define how this may help with the development of new drug targets to induce regeneration.

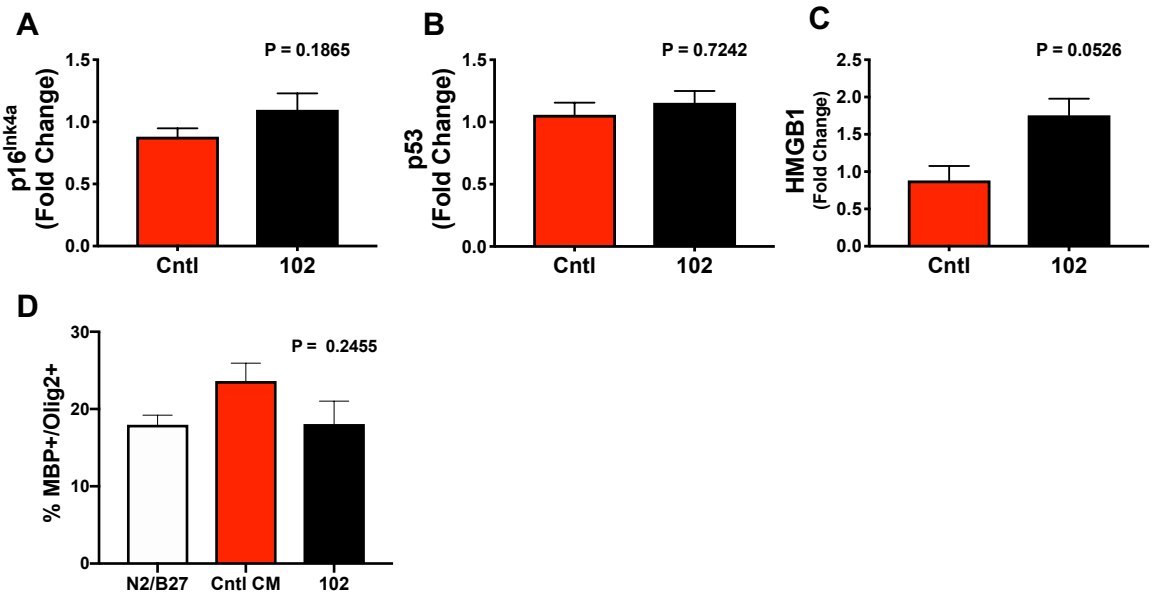


Figure 6.2. No change in senescent markers in neural progenitor cells from an RRMS patient line. *(A, B, C)* $p16^{Ink4a}$, p53, and HMGB1 mRNA expression by qPCR in control NPC and RRMS line 102 NPC cultures revealed no significant changes (unpaired t tests). All qPCR data normalized to control NPC lines. *(D)* Quantitative analysis of oligodendrocyte maturation (MBP+/Olig2+) resulting from culture of OPCs in either N2/B27 (unconditioned NPC media), control NPC CM, or line 102 RRMS NPC CM after 48 hours. No significant changes in OPC differentiation were identified between the conditions (ANOVA).

IV. Application of this Data for the Development of New Therapeutics

The data presented in my thesis opens the potential for new therapeutic avenues to pursue in patients with progressive forms of MS. Currently, there is only one drug that has been approved by the FDA to treat PPMS, ocrelizumab. This recombinant antibody selectively targets B cells. However, the data supporting its benefits are limited: compared to the placebo group, those taking ocrelizumab had a 24% decrease in disability progression after 12 weeks [25]. Progression was defined as an increase in the EDSS score of at least 1 point compared to that of baseline that was sustained after subsequent visits for at least 12 weeks. This study also assayed the patients in their performance on a timed 25-foot walk, the change in total volume of brain lesions on T_2-weighted MRI, change in total brain volume, and change in the Physical Component Summary score of the Medical Outcomes Study 36-Item Short-Form Health Survey, which all were assayed to define progression of the disease [25]. No significant changes were seen in the Physical Component Summary scores in the two groups after 120 weeks, but significant decreases (3.4% decrease compared to baseline) were seen in the total volume of brain lesions, and overall brain-volume loss in the ocrelizumab treated group [25]. That only a single drug may be useful for PPMS patients leaves significant room for improvement in drug therapies to slow progression.

New remyelinating therapies, such as clemastine, benztropine, clobetasol, and miconazole have been proposed to promote OPCs to differentiate and remyelinate lesion areas, but the identification of these compounds did not take into account that the microenvironment of the MS brain may affect the ability to translate these findings to patients [110, 163, 164]. Data I presented in Chapter 4 demonstrated that a screen which encapsulates the nuances of lesions, specific to individuals may impact the quest for potential remyelinating drug candidates. Here, I treated OPCs with conditioned media from the individual PPMS patient lines and demonstrated that each of the new remyelinating drugs have a patient dependent effect in promoting differentiation of OPCs [111]. A combinatorial approach may be beneficial in treating patients with

progressive MS. For example, a combination of drugs that target and reverse senescence along with remyelinating therapeutics may be warranted. As the technology for the development of autologous stem cells becomes more rapid and cost-effective the screening of patient cells and how they respond to specific drugs may be an opportunity to develop catered therapies in the hopes of slowing progression. Clemastine has been tested in patients with RRMS along with a chronic demyelinating optic neuropathy, where it has been shown to induce remyelination of the optic nerve, but a more in-depth clinical trial is needed to determine if it can manage and delay MS progression [165].

The progressive forms of MS have always been associated with an increase in age, but for the first time my thesis studies show the functional consequences and identify NPCs in the disease as senescent. This discovery of senescent cells inhibiting the differentiation of OPCs in the lesion area opens up new therapeutic avenues. Rapamycin was found to reverse senescence of PPMS NPCs and restore their normal functions in supporting OPC differentiation. Rapamycin is used for its immunosuppressive effects but has never been used to target cellular senescence in patients with progressive MS. A recent clinical trial from Iran demonstrated that after 6 months of rapamycin treatment in patients with RRMS there was a decrease in lesion area size measured by MRI due to a decrease in immune cell populations [406]. While we know that rapamycin acts on mTOR signaling, it can have varying impacts on cells. For instance, even though there is only a small immune component to progressive MS, it may be beneficial through the reversal of senescence in NPCs. Senescent NPCs were also found to secrete HMGB1, a pro-inflammatory alarmin, that directly inhibited the differentiation of OPCs. Through the use of a blocking antibody in the PPMS NPC CM the ability of OPCs to differentiate was improved. Determining where HMGB1 binds onto OPCs, and how exactly it is inhibiting differentiation could discover a mechanism by which to target remyelination. HMGB1 binds to RAGE, TLR2, and TLR4 which have all be found to be expressed by OPCs, but the exact downstream mechanism is unknown [407, 408]. Total RNA sequencing of OPCs after treatment with PPMS CM and PPMS CM with

HMGB1 blocking antibody revealed significant differences in epigenetic regulators, demonstrating that HMGB1 has a substantial effect on the development of OPCs. Further experiments delving into the exact genetic changes, and when they occur in OPCs, can help better explain why differentiation is halted in the presence of PPMS NPC conditioned media. Sequestering HMGB1 in lesion areas, inhibiting its effect on OPCs, and reversing senescence in NPCs could possibly enhance regeneration and slow progression of the disease.

Drugs are currently being developed to eliminate senescent cells, called senolytics, to restore natural regeneration. A combinatorial approach using dasatinib (D) and quercetin (Q) has been found to specifically eliminate senescent cells in multiple tissues of aged mice, reduce inflammation, and increase health- and lifespan [264, 267]. The overall effects of senolytic therapy on the aged human CNS is currently unknown, but in a mouse model of Alzheimer's clearance of senescent cells through transgenic targeting ameliorated neurodegeneration [231]. The elimination of senescent NPCs may be beneficial in patients with progressive MS by decreasing inflammation and allowing for regeneration. Currently, the side effects of eliminating a specific cell population through apoptosis in the CNS is unknown and a better understanding is merited before human clinical trials.

Since the development of stem cells their use in transplantation has been studied, especially in neurodegenerative diseases. Work by the Pluchino group has demonstrated that NPCs possess therapeutic potential that is distinct from that of small molecules, as they can sense diverse signals, migrate to specific biological niches, and execute complex behaviors [147, 149, 331]. NPCs have been shown to graft and replace lost cells when transplanted into mouse models of demyelination, but also act by buffering inflammation and stimulating endogenous remyelination [149, 152]. To date, only HSCT has been used in patients with MS, where only a small number of patients benefited from the transplant [176]. Transplantation of NPCs may provide a greater impact by repairing lesion area by buffering inflammation and through the secretion of promyelinating factors [151]. Based on the data presented in this thesis autologous

transplant in patients with progressive MS will not be beneficial due to the senescent phenotype. Autologous transplant avoids the possibility of rejection, but may not be feasible in patients with progressive MS. If specific mutations are identified within the progressive MS NPCs they could be targeted using CRISPR gene editing technology before transplantation. Still, these technologies are expensive and take time, which limits their use in the clinical setting. On the other hand, if healthy NPC transplants in progressive MS patients are found to be beneficial, the generation of a large pool of NPCs from healthy donors could be stock pilled for transplantation. We still have a long way to go before NPC transplants can be used in the clinic as their efficacy and safety in humans is still unknown, and the production of a mass scale of NPCs would be costly, as would the quality control of each batch. Studying how transplantation of NPCs into mouse models of MS ameliorates disease can help uncover new targetable mechanisms to modulate disease progression.

V. Concluding Statement

My thesis demonstrates for the first time a cell autonomous defect in iPS-NPCs derived from patients with primary progressive MS. These cells failed to promote remyelination and provide neuroprotection in a demyelinated mouse model due to a senescent phenotype. Senescent NPCs were identified in post-mortem progressive MS tissue, associated with HMGB1, a pro-inflammatory factor that inhibits OPC differentiation. Rapamycin treatment ameliorated the senescent phenotype in the NPCs, allowing for oligodendrocyte differentiation. This research opens up new therapeutic avenues to pursue in the treatment of progressive MS, such as targeting senescent NPCs to promote remyelination. These data implicate cellular aging and senescence as a process that contributes to remyelination failure in progressive MS which may impact how this disease is modeled and inform development of future myelin regeneration strategies.

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